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(54) Title: HUMANIZED ANTIBODIES TO HUMAN gp39, COMPOSITIONS CONTAINING AND THERAPEUTIC USE THEREOF

(57) Abstract

The present invention is directed to humanized antibodies which bind human gp39 and their use as therapeutic agents. These humanized antibodies are especially useful for treatment of autoimmune diseases; and an immunosuppressant during transplantation of heterologous cells, tissues or organs, cell therapy, and gene therapy.

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HUMANIZED ANTIBODIES TO HUMAN gp39, COMPOSITIONS CONTAINING AND THERAPEUTIC USE THEREOF

FIELD OF THE INVENTION

The present invention is directed to humanized antibodies specific for human gp39, DNA encoding such antibodies, methods for their production, pharmaceutical compositions containing, and the use of such humanized antibodies as therapeutic agents. These antibodies have particular application in the treatment of autoimmune diseases including, e.g., rheumatoid arthritis, multiple sclerosis, diabetes, and systemic lupus erythematosus as well as non-autoimmune diseases including, e.g., graft-versus-host disease and for preventing graft rejection.

BACKGROUND OF THE INVENTION

The immune system is capable of producing two types of antigen-specific responses to foreign antigens. Cell-mediated immunity is the term used to refer to effector functions of the immune system mediated by T lymphocytes. Humoral immunity is the term used to refer to production of antigen-specific antibodies by B lymphocytes. It has long been appreciated that the development of humoral immunity against most antigens requires not only antibody-producing B lymphocytes but also the involvement of helper T (hereinafter Th) lymphocytes. (Mitchison, *Eur. J. Immunol.*, 1:18-25 (1971); Claman and Chaperon, *Transplant Rev.*, 1:92-119 (1969); Katz et al, *Proc. Natl. Acad. Sci. USA*, 70:2624-2629 (1973); Reff et al, *Nature*, 226:1257-1260 (1970)). Certain signals, or "help", are provided by Th cells in response to stimulation by Thymus-dependent (hereinafter TD) antigens. While some B lymphocyte help is mediated by soluble molecules released by Th cells (for instance

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lymphokines such as IL-4 and IL-5), activation of B cells
also requires a contact-dependent interaction between B
cells and Th cells. (Hirohata et al, *J. Immunol.*,
140:3736-3744 (1988); Bartlett et al, *J. Immunol.*,

5   143:1745-1765 (1989)). This indicates that B cell activa-
tion involves an obligatory interaction between cell sur-
face molecules on B cells and Th cells. Such an interac-
tion is further supported by the observation that isolated
plasma membranes of activated T cells can provide helper

10 functions necessary for B cell activation. (Brian, *Proc.
Natl. Acad. Sci. USA*, 85:564-568 (1988); Hodgkin et al, *J.
Immunol.*, 145:2025-2034 (1990); Noelle et al, *J. Immunol.*,
146:1118-1124 (1991)).

It is further known that in a contact-dependent pro-
15 cess termed "T cell helper function", CD4^{+} T lymphocytes
direct the activation and differentiation of B lymphocytes
and thereby regulate the humoral immune response by modu-
lating the specificity, secretion and isotype-encoded
functions of antibody molecules (Mitchell et al, *J. Exp.*

20 *Med.*, 128:821 (1968); Mitchison, *Eur. J. Immunol.*, 1:68
(1971); White et al, *J. Exp. Med.*, 14:664 (1978); Reinherz
et al, *Proc. Natl. Acad. Sci. USA*, 74:4061 (1979); Janeway
et al, *Immunol. Rev.*, 101:39 (1988); O'Brien et al, *J.
Immunol.*, 141:3335 (1988); Rahemtulla et al, *Nature*,

25 353:180 (1991); and Grusby et al, *Science*, 253:1417
(1991)).

The process by which T cells help B cells to differen-
tiate has been divided into two distinct phases; the induc-
tive and effector phases (Vitetta et al, *Adv. Immunol.*,

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45:1 (1989); Noelle et al, *Immunol. Today*, 11:361 (1990)).

In the inductive phase, resting T cells contact antigen-

primed B cells and this association allows clonotypic T

cell receptor (TCR)-CD4 complexes to interact with Ia/Ag

5   complexes on B cells (Janeway et al, *Immunol. Rev.*, 101:39

(1988); Katz et al, *Proc. Natl. Acad. Sci.*, 70:2624 (1973);

Zinkernagel, *Adv. Exp. Med.*, 66:527 (1976); Sprent, *J. Exp.*

Med., 147:1159 (1978); Sprent, *Immunol. Rev.*, 42:158

(1978); Jones et al, *Nature*, 292:547 (1981); Julius et al,

10   *Eur. J. Immunol.*, 18:375 (1982); Chestnut et al, *J.*

Immunol., 126:1575 (1981); and Rogozinski et al, *J.*

Immunol., 126:735 (1984)). TCR/CD4 recognition of Ia/Ag

results in the formation of stable T-B cognate pairs and

bi-directional T and B cell activation (Sanders et al, *J.*

15   *Immunol.*, 137:2395 (1986); Snow et al, *J. Immunol.*, 130:614

(1983); Krusemeier et al, *J. Immunol.*, 140:367 (1988);

Noelle et al, *J. Immunol.*, 143:1807 (1989); Bartlett et al,

J. Immunol., 143:1745 (1989); and Kupfer et al, *Annu. Rev.*

Immunol., 7:309 (1987)). In the effector phase, activated

20   T cells drive B cell differentiation by secreting lympho-

kines (Thompson et al, *J. Immunol.*, 134:369 (1985)) and by

contact-dependent stimuli (Noelle et al, *J. Immunol.*,

143:1807 (1989); Clement et al, *J. Immunol.*, 140:3736

(1984); Crow et al, *J. Exp. Med.*, 164:1760 (1986); Brian,

25   *Proc. Natl. Acad. Sci.*, USA, 85:564 (1988); Hirohata et al,

J. Immunol. 140:3736 (1988); Jover et al, *Clin. Immunol.*

Immun., 53:90 (1989); Whalen et al, *J. Immunol.*, 141:2230

(1988); Pollok et al, *J. Immunol.*, 146:1633 (1991); and

Bartlett et al, *J. Immunol.*, 143:1745 (1990)), both of

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which are required for T cells to drive small resting B
cells to terminally differentiate into Ig secreting cells
(Clement et al, *J. Immunol.*, 132:740 (1984); Martinez
et al, *Nature*, 290:60 (1981); and Andersson et al, *Proc.*
5   *Natl. Acad. Sci., USA*, 77:1612 (1980)).

Although the inductive phase of T cell help is Ag-
dependent and MHC-restricted (Janeway et al, *Immun. Rev.*,
101:34 (1988); Katz et al, *Proc. Natl. Acad. Sci., USA*,
10:2624 (1973); Zinkernagle, *Adv. Exp. Med. Biol.*, 66:527
10   (1976)); the effector phase of T cell helper function can
be Ag-independent and MHC-nonrestricted (Clement et al, *J.*
Immunol., 132:740 (1984); Hirohata et al, *J. Immunol.*,
140:3736 (1988); Whalen et al, *J. Immunol.*, 143:1715
(1988)). An additional contrasting feature is that the
15   inductive phase of T cell help often requires CD4 molecules
and is inhibited by anti-CD4 mAb (Rogozinski et al, *J.*
Immunol., 126:735 (1984)), whereas helper effector function
does not require CD4 molecules (Friedman et al, *Cell*
Immunol., 103:105 (1986)) and is not inhibited by anti-CD4
20   mAbs (Brian, *Proc. Natl. Acad. Sci., USA*, 85:564 (1988);
Hirohata et al, *J. Immunol.*, 140:3736 (1988); Whalen et al,
J. Immunol., 143:1745 (1988); and Tohma et al, *J. Immunol.*,
146:2547 (1991)). The non-specific helper effector func-
tion is believed to be focused on specific B cell targets
25   by the localized nature of the T-B cell interactions with
antigen specific, cognate pairs (Bartlett et al, *J.*
Immunol., 143:1745 (1989); Kupfer et al, *J. Exp. Med.*,
165:1565 (1987) and Poo et al, *Nature*, 332:378 (1988)).

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Although terminal B cell differentiation requires both
contact- and lymphokine-mediated stimuli from T cells,
intermediate stages of B cell differentiation can be in-
duced by activated T cell surfaces in the absence of se-
5   creted factors (Crow et al, *J. Exp. Med.*, 164:1760 (1986);
Brian, *Proc. Natl. Acad. Sci., USA*, 85:564 (1988); Sekita
et al, *Eur. J. Immunol.*, 18:1405 (1988); Hodgkin et al, *J.
Immunol.*, 145:2025 (1990); Noelle et al, *FASEB J,* 5:2770
(1991)). These intermediate effects on B cells include
10   induction of surface CD23 expression (Crow et al, *Cell
Immunol.*, 121:94 (1989)), enzymes associated with cell
cycle progression (Pollok et al, *J. Immunol.*, 146:1633
(1991)) and responsiveness to lymphokines (Noelle et al,
FASEB J, 5:2770 (1989); Pollok et al, *J. Immunol.*, 146:1633
15   (1991)). Recently some of the activation-induced T cell
surface molecules that direct B cell activation have been
identified. Additionally, functional studies have charac-
terized some features of activation-induced T cell surface
molecules that direct B cell activation. First, T cells
20   acquire the ability to stimulate B cells 4-8 h following
activation (Bartlett et al, *J. Immunol.*, 145:3956 (1990)
and Tohma et al, *J. Immunol.*, 146:2544 (1991)). Second,
the B cell stimulatory activity associated with the surfac-
es of activated T cells is preserved on paraformaldehyde
25   fixed cells (Noelle et al, *J. Immunol.*, 143:1807 (1989);
Cros et al, *J. Exp. Med.*, 164:1760 (1986); Pollok et al, *J.
Immunol.*, 146:1633 (1991); Tohma et al, *J. Immunol.*,
146:2544 (1991); and Kubota et al, *Immunol.*, 72:40 (1991))
and on purified membrane fragments (Hodgkin et al, *J.*

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Immunol., 145:2025 (1990) and Martinez et al, *Nature*, 290:60 (1981)). Third, the B cell stimulatory activity is sensitive to protease treatment (Noelle et al, *J. Immunol.*, 143:1807 (1989); Sekita et al, *Eur. J. Immunol.*, 18:1405

5   (1988); and Hodgkin et al, *J. Immunol.*, 145:2025 (1990). Fourth, the process of acquiring these surface active structures following T cell activation is inhibited by cycloheximide (Tohma et al, *J. Immunol.*, 196:2349 (1991) and Hodgkin et al, *J. Immunol.*, 195:2025 (1990)).

10   A cell surface molecule, CD40, has been identified on immature and mature B lymphocytes which, when crosslinked by antibodies, induces B cell proliferation. Valle et al, *Eur. J. Immunol.*, 19:1463-1467 (1989); Gordon et al, *J. Immunol.*, 140:1425-1430 (1988); Gruder et al, *J. Immunol.*,

15   142:4144-4152 (1989).

         CD40 has been molecularly cloned and characterized (Stamenkovic et al, *EMBO J.*, 8:1403-1410 (1989)).

         CD40 is expressed on B cells, interdigitating dendrit-ic cells, macrophages, follicular dendritic cells, and

20   thymic epithelium (Clark, *Tissue Antigens* 36:33 (1990); Alderson et al, *J. Exp. Med.*, 178:669 (1993); Galy et al, *J. Immunol.* 142:772 (1992)). Human CD40 is a type I mem-brane protein of 50 kDa and belongs to the nerve growth factor receptor family (Hollenbaugh et al, *Immunol. Rev.*,

25   138:23 (1994)). Signaling through CD40 in the presence of IL-10 induces IgA, IgM and IgG production, indicating that isotype switching is regulated through these interactions. The interaction between CD40 and its ligand results in a

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primed state of the B cell, rendering it receptive to
subsequent signals.

Also, a ligand for CD40, gp39 (also called CD40 ligand
or CD40L) has recently been molecularly cloned and charac-
5   terized (Armitage et al, *Nature*, 357:80-82 (1992); Lederman
et al, *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh
et al, *EMBO J.*, 11:4313-4319 (1992)). The gp39 protein is
expressed on activated, but not resting, CD4+ Th cells.
Spriggs et al, *J. Exp. Med.*, 176:1543-1550 (1992); Lane
10   et al, *Eur. J. Immunol.*, 22:2573-2578 (1992); and Roy
et al, *J. Immunol.*, 151:1-14 (1993). Cells transfected
with gp39 gene and expressing the gp39 protein on their
surface can trigger B cell proliferation and, together with
other stimulatory signals, can induce antibody production.
15   Armitage et al, *Nature*, 357:80-82 (1992); and Hollenbaugh
et al, *EMBO J.*, 11:4313-4319 (1992). In particular, the
ligand for CD40, gp39, has been identified for the mouse
(Noelle et al, *Proc. Natl. Acad. Sci. USA*, 89:6550 (1992);
Armitage et al, *Nature*, 357:80 (1992)) and for humans
20   (Hollenbaugh et al, *Embo. J.* 11:4313 (1992); Spriggs et al,
J. Exp. Met., 176:1543 (1992)). gp39 is a type II membrane
protein and is part of a new gene super family which in-
cludes TNF-α, TNF-β and the ligands for FAS, CD27, CD30 and
4-1BB.

25   Expression of gp39 can be readily induced *in vitro* on
CD4+ T cells using either anti-CD3 antibody or phorbol
myristate acetate (PMA) plus ionomycin. Expression is
rapid and transient, peaking at 6-8 hours and returning to
near resting levels between 24 and 48 hours (Roy et al, *J.*

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Immunol., 151:2497 (1993)). *In vivo*, gp39 has been report-
ed in humans to be present on CD4⁺ T cells in the mantle and
centrocytic zones of lymphoid follicles and the periarter-
iolar lymphocyte sheath of the spleen, in association with

5   CD40⁺ B cells (Lederman et al, *J. Immunol.*, 149:3807
(1992)). gp39⁺ T cells produce IL-2, IL-4 and IFN-γ (Van
der Eetwegh et al, *J. Exp. Med.*, 178:1555 (1993)).

Unique insights into the novel role of gp39 in the
regulation of humoral immunity have been provided by stud-

10   ies of a human disease, X-linked hyper-IgM syndrome (HIM).
HIM is a profound, X-linked immunodeficiency typified by a
loss in thymus dependent humoral immunity, the inability to
produce IgG, IgA and IgE. Mutations in the gp39 gene were
responsible for the expression of a non-functional gp39

15   protein and the inability of the helper T cells from HIM
patients to activate B cells (Allen et al, *Science*, 259:990
(1993); Aruffo et al, *Cell*, 72:291 (1993); DiSanto et al,
Nature, 361:541 (1993); Korthauer et al, *Nature*, 361:539
(1993)). These studies support the conclusion that early

20   after T cell receptor engagement of the peptide/MHC class
II complex, gp39 is induced on the cognate helper T cell,
and the binding of gp39 to CD40 on the B cell induces the B
cell to move into the cell cycle and differentiate to
immunoglobulin (Ig) secretion and isotype switching.

25   Functional studies have shown that treatment of mice
with anti-gp39 completely abolished the antibody response
against thymus dependent antigens (SRBC and TNP-KLH), but
not thymus independent antigens (TNP-Ficoll) (Foy et al, *J.
Exp. Med.*, 178:1567 (1993)). In addition, treatment with

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anti-gp39 prevented the development of collagen-induced arthritis (CIA) in mice injected with collagen (Durie et al, *Science*, 261:1328 (1993)). Finally, anti-gp39 pre-vented formation of memory B cells and germinal centers in

5   mouse spleen (Foy et al, *J. Exp. Med.*, 180:157 (1994)). Collectively, these data provide extensive evidence that the interaction between gp39 on T cells and CD40 on B cells is essential for antibody responses against thymus depen-dent antigens.

10   Recently, a number of murine models of autoimmune disease have been exploited to evaluate the potential therapeutic value of anti-gp39 administration on the devel-opment of disease. A brief discussion of the results of studies in these models are provided below:

15 *Collagen-Induced Arthritis:*

CIA is an animal model for the human autoimmune dis-ease rheumatoid arthritis (RA) (Trenthorn et al, *J. Exp. Med.*, 146:857 (1977)). This disease can be induced in many species by the administration of heterologous type II

20   collagen (Courtenay et al, *Nature*, 283:665 (1980); Cathcart et al, *Lab. Invest.*, 54:26 (1986)).

To study the effect anti-gp39 on the induction of CIA (Durie et al, *Science*, 261:1328 (1993)) male DBA1/J mice were injected intradermally with chick type II collagen

25   emulsified in complete Freund's adjuvant at the base of the tail. A subsequent challenge was carried out 21 days later. Mice were then treated with the relevant control antibody or anti-gp39. Groups of mice treated with anti-gp39 showed no titers of anti-collagen antibodies compared

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to immunized, untreated control mice. Histological analy-
sis indicated that mice treated with anti-gp39 antibody
showed no signs of inflammation or any of the typical
pathohistological manifestations of the disease observed in
5   immunized animals. These results indicated that gp39-CD40
interactions are absolutely essential in the induction of
CIA. If the initial cognate interaction between the T cell
and B cell is not obtained, then the downstream processes,
such as autoantibody formation and the resulting inflamma-
10   tory responses, do not occur.

Recently it has been shown that gp39 is important in
activating monocytes to produce TNF-α and IL-6 in the
absence of GM-CSF, IL-3 and IFN-γ (Alderson et al, *J. Exp.
Med.*, 178:669 (1993)). TNF-α has been implicated in the
15   CIA disease process (Thorbecke et al, *Eur. J. Immunol.*,
89:7375 (1992) and in RA (DiGiovane et al, *Ann. Rheum.
Dis.*, 47:68 (1988); Chu et al, *Arthrit. Rheum.*, 39:1125
(1991); Brennan et al, *Eur. J. Immunol.*, 22:1907 (1992).
Thus, inhibition of TNF-α by anti-gp39 may have profound
20   anti-inflammatory effects in the joints of arthritic mice.
Both inhibition of TNF-α and of T cell-B cell interactions
by anti-gp39 may be contributory to manifestations of CIA.
Experimental Allergic Encephalomyelitis (EAE):

EAE is an experimental autoimmune disease of the
25   central nervous system (CNS) (Zamvil et al, *Ann. Rev.
Immunol.*, 8:579 (1990) and is a disease model for the human
autoimmune condition, multiple sclerosis (MS) (Alvord et
al, "Experimental Allergic Model for Multiple Sclerosis,"
NY 511 (1984)). It is readily induced in mammalian species

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by immunizations of myelin basic protein purified from the CNS or an encephalitogenic proteolipid (PLP). SJL/J mice are a susceptible strain of mice ($H-2^s$) and, upon induction of EAE, these mice develop an acute paralytic disease and

5   an acute cellular infiltrate is identifiable within the CNS.

Classen and co-workers (unpublished data) have studied the effects of anti-gp39 on the induction of EAE in SJL/J mice. They found that EAE development was completely

10   suppressed in the anti-gp39 treated animals. In addition, anti-PLP antibody responses were delayed and reduced compared to those obtained for control animals.

EAE is an example of a cell-mediated autoimmune disease mediated via T cells, with no direct evidence for the

15   requirement for autoantibodies in disease progression. Interference with the interaction between gp39 and CD40 prevents disease induction and the adoptive transfer of disease.

Chronic (c) and acute (a) graft-versus-host-disease (GVHD):

20   Chronic and acute GVHD result from donor cells responding to host disparate MHC alleles. In cGVHD ($H-2^d$-->$H-2^{bd}$), heightened polyclonal immunoglobulin production is due to the interaction of allospecific helper T cells and the host B cells. *In vivo* administration of anti-gp39 antibody

25   blocked cGVHD-induced serum anti-DNA autoantibodies, IgE production, spontaneous immunoglobulin production *in vitro*, associated splenomegaly and the ability to transfer disease. Durie F.H. et al, *J. Clin. Invest.*, 94:133 (1994). Antibody production remained inhibited for extended periods

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of time after termination of anti-gp39 administration.
Anti-allogeneic cytotoxic T lymphocyte (CTL) responses
induced in GVHD were also prevented by the *in vivo* admin-
istration of anti-gp39. These data suggest that CD40-gp39

5   interactions are critical in the generation of both forms
of GVHD. The fact that CTL responses were inhibited and a
brief treatment with anti-gp39 resulted in long-term pre-
vention of disease suggest permanent alterations in the T
cell compartment by the co-administration of allogeneic

10   cells and anti-gp39 antibody.

Various research groups have reported the production
of murine antibodies specific to gp39, which are disclosed
to possess therapeutic utility as immunosuppressants. For
example, WO 93/09812, published May 27, 1993, and assigned

15   to Columbia University; EP 0,555,880, published August 18,
1993, and PCT US/94/09872, filed September 2, 1994 by
Noelle et al and assigned to Dartmouth College, describe
murine antibodies specific to gp39 and their use as thera-
peutics and immunosuppressants.

20   Also, Scaria et al, *Gene Therapy*, 4:611-617 (1997)
report the use of an antibody to gp39 to inhibit humoral
and cellular immune responses to a DNA (adenoviral/vector).

However, while murine antibodies have applicability as
therapeutic agents in humans, they are disadvantageous in

25   some respects. Specifically, murine antibodies, because of
the fact that they are of foreign species origin, may be
immunogenic in humans. This often results in a neutraliz-
ing antibody response, which is particularly problematic if
the antibodies are desired to be administered repeatedly,

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e.g., in treatment of a chronic or recurrent disease condi-
tion. Also, because they contain murine constant domains
they may not exhibit human effector functions.

In an effort to eliminate or reduce such problems,
5   chimeric antibodies have been disclosed. Chimeric antibod-
ies contain portions of two different antibodies, typically
of two different species. Generally, such antibodies
contain human constant and another species, typically
murine variable regions. For example, some mouse/human
10 chimeric antibodies have been reported which exhibit bind-
ing characteristics of the parental mouse antibody, and
effector functions associated with the human constant
region. See, e.g., Cabilly et al, U.S. Patent 4,816,567;
Shoemaker et al., U.S. Patent 4,978,745; Beavers et al.,
15 U.S. Patent 4,975,369; and Boss et al., U.S. Patent
4,816,397, all of which are incorporated by reference
herein. Generally, these chimeric antibodies are con-
structed by preparing a genomic gene library from DNA
extracted from pre-existing murine hybridomas (Nishimura
20 et al, Cancer Research, 47:999 (1987)). The library is
then screened for variable region genes from both heavy and
light chains exhibiting the correct antibody fragment
rearrangement patterns. Alternatively, cDNA libraries are
prepared from RNA extracted from the hybridomas and
25 screened, or the variable regions are obtained by polymer-
ase chain reaction. The cloned variable region genes are
then ligated into an expression vector containing cloned
cassettes of the appropriate heavy or light chain human
constant region gene. The chimeric genes are then

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expressed in a cell line of choice, usually a murine myelo-
ma line. Such chimeric antibodies have been used in human
therapy.

In a commonly assigned application, Serial No.

5   07/912,292, "Primatized"™ antibodies are disclosed which
contain human constant and Old World monkey variable re-
gions. These Primatized™ antibodies are well tolerated in
humans given their low or weak immunogenicity.

Also, humanized antibodies are known in the art.

10   Ideally, "humanization" results in an antibody that is less
immunogenic, with complete retention of the antigen-binding
properties of the original molecule. In order to retain
all the antigen-binding properties of the original anti-
body, the structure of its combining-site has to be faith-

15   fully reproduced in the "humanized" version. This can
potentially be achieved by transplanting the combining site
of the nonhuman antibody onto a human framework, either
(a) by grafting the entire nonhuman variable domains onto
human constant regions to generate a chimeric antibody

20   (Morrison et al, *Proc. Natl. Acad. Sci., USA*, 81:6801
(1984); Morrison and Oi, *Adv. Immunol.*, 44:65 (1988) (which
preserves the ligand-binding properties, but which also
retains the immunogenicity of the nonhuman variable do-
mains); (b) by grafting only the nonhuman CDRs onto human

25   framework and constant regions with or without retention of
critical framework residues (Jones et al, *Nature*, 321:522
(1986); Verhoeyen et al, *Science*, 239:1539 (1988)); or (c)
by transplanting the entire nonhuman variable domains (to
preserve ligand-binding properties) but also "cloaking"

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them with a human-like surface through judicious replace-
ment of exposed residues (to reduce antigenicity) (Padlan,
Molec. Immunol., 28:489 (1991)).

Essentially, humanization by CDR grafting involves
5   transplanting only the CDRs onto human fragment onto human
framework and constant regions. Theoretically, this should
substantially eliminate immunogenicity (except if allotypic
or idiotypic differences exist). However, it has been
reported that some framework residues of the original
10   antibody also need to be preserved (Riechmann et al, *Na-
ture*, 332:323 (1988); Queen et al, *Proc. Natl. Acad. Sci.
USA*, 86:10,029 (1989)).

The framework residues which need to be preserved can
be identified by computer modeling. Alternatively, criti-
15   cal framework residues may potentially be identified by
comparing known antibody combining site structures (Padlan,
Molec. Immun., 31(3):169-217 (1994)).

The residues which potentially affect antigen binding
fall into several groups. The first group comprises resi-
20   dues that are contiguous with the combining site surface
which could therefore make direct contact with antigens.
They include the amino-terminal residues and those adjacent
to the CDRs. The second group includes residues that could
alter the structure or relative alignment of the CDRs
25   either by contacting the CDRs or the opposite chains. The
third group comprises amino acids with buried side chains
that could influence the structural integrity of the vari-
able domains. The residues in these groups are usually
found in the same positions (Padlan, 1994 (*Id.*) according

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to the adopted numbering system (see Kabat et al, "Sequenc-
es of proteins of immunological interest, 5th ed., Pub. No.
91-3242, U.S. Dept. Health & Human Services, NIH, Bethesda,
MD, 1991).

5   However, while humanized antibodies are desirable
because of their potential low immunogenicity in humans,
their production is unpredictable. For example, sequence
modification of antibodies may result in substantial or
even total loss of antigen binding function, or loss of
10   binding specificity. Alternatively, "humanized antibodies"
may still exhibit immunogenicity in humans, irrespective of
sequence modification.

Thus, there still exists a significant need in the art
for novel humanized antibodies to desired antigens. More
15   specifically, there exists a need in the art for humanized
antibodies specific to gp39, because of their potential as
immunotherapeutic agents.

OBJECTS OF THE INVENTION

Toward this end, it is an object of the invention to
20   provide humanized antibodies which are specific to human
gp39.

More specifically, it is an object of the invention to
provide humanized antibodies derived from murine antibodies
to gp39 and in particular 24-31, a specific murine antibody
25   which binds to human gp39.

It is also an object of the invention to provide
pharmaceutical compositions containing humanized antibodies
which are specific to human gp39.

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It is a more specific object of the invention to provide pharmaceutical compositions containing humanized antibodies derived from 24-31, a murine antibody which specifically binds to human gp39.

5   It is another specific object of the invention to provide methods of using humanized antibodies to human gp39 for treatment of human disease conditions, which are treatable by modulation of gp39 expression and/or inhibition of the gp39/CD40 binding interaction including, e.g., autoim-

10   mune diseases such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, idiopathic thrombocytopenic purpura (ITP), diabetes and non-autoimmune conditions such as graft-versus-host disease and transplantation.

It is still another object of the invention to provide

15   nucleic acid sequences which encode for humanized antibodies to human gp39.

It is a more specific object of the invention to provide nucleic acid sequences which encode humanized antibodies derived from 24-31, a murine antibody which

20   specifically binds to human gp39 antigen.

It is another object of the invention to provide vectors which provide for the expression of humanized antibodies to human gp39, in particular humanized antibodies derived from 24-31, a murine antibody which specifical-

25   ly binds to human gp39 antigen.

SUMMARY OF THE INVENTION

In its broadest embodiment, the present invention is directed to humanized antibodies which retain not less than about one-tenth and more preferably not lower than

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one-third the gp39 antigen binding affinity of the murine
24-31 antibody and/or which retain not less than about one-
tenth and more preferably not less than about one-third the
in vitro functional activity of the murine antibody 24-31,
5   e.g., in B-cell assays which measure T-cell dependent
antibody production. More particularly, the present human-
ized antibodies retain at least one-tenth and more prefera-
bly at least about one-third the half-maximal potency in *in
vitro* functional activity in a B cell assay at a concentra-
10   tion of not more than three times the concentration of the
24-31 antibody.

The present invention is further directed to humanized
antibodies which bind to the same epitope as the murine 24-
31 antibody and/or which are capable of competing with the
15   murine 24-31 antibody for inhibiting the binding of CD40 to
gp39 and/or which contain the CDR's of the 24-31 antibody.

The present invention is more preferably directed to
humanized antibodies derived from murine 24-31 which pos-
sess the humanized variable light sequences and/or human-
20   ized variable heavy sequences set forth below:

(1)   DIVMTQSPSFLSASVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSLQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(2)   DIVMTQSPDSLAVSLGERATINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSLQAEDVADYFC QQYNSYPYT FGGGTKLEIK;

25   (3)   DIVMTQSPSFMSTSVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSMQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(4)   DIVMTQSPDSMATSLGERVTINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSMQAEDVADYFC QQYNSYPYT FGGGTKLEIK

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and a humanized variable heavy sequence selected from the following group:

(1)   EVQLQESGPGLVKPSETLSLTCTVSGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

5   (2)   EVQLQESGPGLVKPSQTLSLTCTVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(3)   EVQLQESGPGLVKPSQTLSLTCAVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSNNQFSLNLNSVTRADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

10 (4)   EVQLQESGPGLVKPSETLSLTCAVYGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFYLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS

as well as variants and equivalents thereof. Variants and equivalents thereof in the present invention are intended to embrace humanized immunoglobulin sequences wherein one

15 or several of the amino acid residues in the above identified humanized variable heavy and/or variable light sequences are modified by substitution, addition and/or deletion in such manner that does not substantially effect gp39 antigen binding affinity. In particular, the present

20 invention embraces variants and equivalents which contain conservative substitution mutations, i.e., the substitution of one or more amino acids by similar amino acids. For example, conservative substitution refers to the substitution of an amino acid within the same general class, e.g.,

25 an acidic amino acid, or a basic amino acid, a neutral amino acid by another amino acid within the same class.

- 20 -

What is intended by a conservative amino acid substitution
is well known in the art. Preferably, such variants and
equivalents will retain not less than about one-tenth and
more preferably not less than about one-third the gp39

5   antigen binding affinity as the parent murine 24-31 anti-
body and more preferably not less than about one-third the
gp39 antigen binding affinity as the murine 24-31 antibody.
Additionally, such variants and equivalents will preferably
retain not lower than one-tenth and more preferably retain

10   at least about one-third the *in vitro* functional activity
of murine antibody 24-31, e.g., in B-cell assays which
measure T-cell dependent antibody production. More prefer-
ably, these variants and equivalents will retain at least
about one-third the *in vitro* functional activity of murine

15   antibody 24-31, for example, in B-cell assays which measure
T-cell dependent antibody production. More specifically,
these antibodies will retain the half-maximal potency in *in
vitro* functional activity in a B cell assay at a concentra-
tion of not more than about three times the concentration

20   of the parent 24-31 antibody.

The present invention is further directed to nucleic
acid sequences which encode for the expression of such
humanized antibodies, as well as expression vectors which
provide for the production of humanized antibodies in

25   recombinant host cells. In the most preferred embodiments
these DNA sequences will encode for the humanized variable
heavy and/or humanized variable light sequences set forth
below:

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(1) DIVMTQSPSFLSASVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSLQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(2) DIVMTQSPDSLAVSLGERATINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSLQAEDVADYFC QQYNSYPYT FGGGTKLEIK;

5 (3) DIVMTQSPSFMSTSVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSMQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(4) DIVMTQSPDSMATSLGERVTINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSMQAEDVADYFC QQYNSYPYT FGGGTKLEIK

and a humanized variable heavy sequence selected from the
10   following group:

(1) EVQLQESGPGLVKPSETLSLTCTVSGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(2) EVQLQESGPGLVKPSQTLSLTCTVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

15 (3) EVQLQESGPGLVKPSQTLSLTCAVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSNNQFSLNLNSVTRADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(4) EVQLQESGPGLVKPSETLSLTCAVYGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFYLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS.

20   Moreover, the present invention also embraces equiva-
lent and variants thereof as defined *supra*.

The present invention is further directed to the use
of the above-identified humanized antibodies specific to

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gp39 as pharmaceuticals. The present invention is also
directed to the use of the subject humanized anti-gp39
antibodies for treating diseases treatable by modulation of
gp39 expression or by inhibition of the gp39/CD40 interac-
5   tion. The present invention is more particularly directed
to the use of humanized antibodies of the above-identified
humanized antibodies specific to gp39 for the treatment of
autoimmune disorders, for example, rheumatoid arthritis,
multiple sclerosis, diabetes, systemic lupus erythematosus
10   and ITP. The present invention is further directed to the
use of the subject humanized antibodies to gp39 for the
treatment of non-autoimmune disorders including graft-
versus-host disease and for inhibiting graft rejection.

Also, the subject invention is further directed to
15   usage of the subject humanized antibodies as immunosuppres-
sants, in particular during gene or cellular therapy. The
subject humanized antibodies should enhance the efficacy of
gene therapy or cellular therapy by inhibiting adverse
immunogenic reaction to vectors and cells used therein.
20   For example, they may be used to inhibit humoral and cellu-
lar immune responses against viral vectors, e.g., retro-
viral vectors, adenoviral vectors. Also, the use of such
antibodies should enable such cells or vectors to be admin-
istered repeatedly, which will facilitate treatment of
25   chronic diseases such as cancers and autoimmune diseases.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the IDEC expression vector N5KG1 used
to express humanized and chimeric antibodies derived from
24-31.

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Figure 2a contains results of a B cell proliferation assay which contacts human PBLs with soluble gp39-CD8, recombinant human IL-4 and the murine 24-31 antibody or control murine IgG1 monoclonal antibody which demonstrate
5   that 24-31 antibody inhibits B cell proliferation induced by gp39.

Figure 2B contains results of B cell differentiation assay using mitomycin treated T cells activated with immobilized anti-CD3 cultured in the present of IGD⁺ B cells and
10   different concentrations of the 24-31 antibody which demonstrate that 24-31 antibody inhibits T-cell dependent polyclonal antibody production by human B cells.

Figure 3 contains FACS of non-transfected CHO cells and a gp39 transfectant.

15   **Figure 4** contains the amino acid sequence and DNA sequence corresponding to a preferred humanized variable light sequence (including the complementarity determining regions) referred to as VL#1 or preferred humanized variable light sequence (1).

20   **Figure 5** contains the amino acid and DNA sequence corresponding to a preferred humanized variable ligand sequence (including the complementarity determining regions) referred to as VL#2 or preferred humanized variable light sequence (2).

25   **Figure 6** contains the amino acid and DNA sequence corresponding to a preferred humanized variable heavy sequence (including the complementarity determining regions) referred to as VH#1 of preferred humanized variable heavy sequence (1).

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Figure 7 contains the amino acid and DNA sequence of the variable light sequence of 24-31 (non-humanized).

Figure 8 contains the amino acid and DNA sequence of the variable heavy sequence of 24-31 (non-humanized).

5   Figure 9 compares binding of murine 24-31, chimeric 24-31 and a humanized 24-31 antibody to gp39 expressing CHO cells.

Figure 10 contains results of a competition assay comparing the binding of 24-31 (biotin) and humanized,

10   chimeric and 24-31 to gp39 expressing CHO cells.

Figure 11 contains results of an assay which measures effects of murine 24-31 and a humanized 24-31 antibody of the invention on human IgM production by B cells cultured in the presence of mitomycin C treated T cells.

15   Figure 12 contains results of an assay comparing binding of two humanized antibodies of the present invention to gp39 expressing CHO cells.

Figure 13 contains the Scatchard plot for murine 24-31.

20   Figure 14 contains the Scatchard plot for humanized Version 1.

Figure 15 contains the Scatchard plot for humanized Version 2.

DETAILED DESCRIPTION OF THE INVENTION

25   Prior to setting forth the invention, definitions of certain terms which are used in this disclosure are set forth below:

Humanized antibody - This will refer to an antibody derived from a non-human antibody, typically murine, that

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retains or substantially retains the antigen-binding prop-
erties of the parent antibody but which is less immunogenic
in humans. This may be achieved by various methods includ-
ing (a) grafting the entire non-human variable domains onto
5   human constant regions to generate chimeric antibodies, (b)
grafting only the non-human CDRs onto human framework and
constant regions with or without retention of critical
framework residues, or (c) transplanting the entire non-
human variable domains, but "cloaking" them with a human-
10   like section by replacement of surface residues. Such
methods are disclosed in Jones et al, Morrison et al, *Proc.
Natl. Acad. Sci.*, 81:6851-6855 (1984); Morrison and Oi,
Adv. Immunol., 44:65-92 (1988); Verhoeyen et al, *Science*,
239:1534-1536 (1988); Padlan, *Molec. Immun.*, 28:489-498
15   (1991); Padlan, *Molec. Immun.*, 31(3):169-217 (1994), all of
which are incorporated by reference.

Complementarity Determining Region, or CDR - The term
CDR, as used herein, refers to amino acid sequences which
together define the binding affinity and specificity of the
20   natural Fv region of a native immunoglobulin binding site
as delineated by Kabat et al (1991).

Framework Region - The term FR, as used herein, refers
to amino acid sequences interposed between CDRs. These
portions of the antibody serve to hold the CDRs in appro-
25   priate orientation (allows for CDRs to bind antigen).

Constant Region - The portion of the antibody molecule
which confers effector functions. In the present inven-
tion, murine constant regions are substituted by human
constant regions. The constant regions of the subject

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chimeric or humanized antibodies are derived from human immunoglobulins. The heavy chain constant region can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu. Further, heavy chains of various

5   subclasses (such as the IgG subclasses of heavy chains) are responsible for different effector functions and thus, by choosing the desired heavy chain constant region, chimeric antibodies with desired effector function can be produced. Preferred constant regions are gamma 1 (IgG1), gamma 3

10   (IgG3) and gamma 4 (IgG4). More preferred is an Fc region of the gamma 1 (IgG1) isotype. The light chain constant region can be of the kappa or lambda type, preferably of the kappa type.

        Chimeric antibody - This is an antibody containing

15   sequences derived from two different antibodies, which typically are of different species. Most typically chimeric antibodies comprise human and murine antibody fragments, generally human constant and murine variable regions.

20   Immunogenicity - A measure of the ability of a targeting protein or therapeutic moiety to elicit an immune response (humoral or cellular) when administered to a recipient. The present invention is concerned with the immunogenicity of the subject humanized antibodies or

25   fragments thereof.

        Humanized or chimeric antibody of reduced immunogenicity - This refers to an antibody or humanized antibody exhibiting reduced immunogenicity relative to the parent antibody, e.g., the 24-31 antibody.

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   <u>Humanized antibody substantially retaining the binding</u>
<u>properties of the parent antibody</u> - This refers to a human-
ized or chimeric antibody which retains the ability to
specifically bind the antigen recognized by the parent
5   antibody used to produce such humanized or chimeric anti-
body. Humanized or chimeric antibodies which substantially
retain the binding properties of 24-31 will bind to human
gp39. Preferably the humanized or chimeric antibody will
exhibit the same or substantially the same antigen-binding
10   affinity and avidity as the parent antibody. Ideally, the
affinity of the antibody will not be less than 10% of the
parent antibody affinity, more preferably not less than
about 30%, and most preferably the affinity will not be
less than 50% of the parent antibody. Methods for assaying
15   antigen-binding affinity are well known in the art and
include half-maximal binding assays, competition assays,
and Scatchard analysis. Suitable antigen binding assays
are described in this application.

   The present invention is directed to novel humanized
20   monoclonal antibodies which bind human gp39 and their use
as therapeutic agents. The present invention is further
directed toward nucleic acid sequences which encode said
humanized antibodies, and their expression in recombinant
host cells.

25   More specifically, the present invention is directed
toward humanized antibodies derived from murine antibody
24-31 which specifically binds to human gp39.

   Murine antibody 24-31 is a murine antibody raised
against human gp39 which functionally inactivates gp39 both

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in vitro and *in vivo*. Therefore, it possesses properties
which render it potentially useful for treatment of diseas-
es wherein gp39 inactivation and/or modulation or inhibi-
tion of the gp39/CD40 interaction is desirable. In partic-

5   ular, such diseases include autoimmune diseases such as,
e.g., rheumatoid arthritis, multiple sclerosis, ITP, diabe-
tes, and systemic lupus erythematosus as well as non-auto-
immune diseases such as graft-versus-host disease and graft
rejection.

10   However, while murine antibody 24-31 possesses func-
tional properties which render it potentially suitable as a
therapeutic agent, it possesses several potential disadvan-
tages. Namely, because it is of murine origin it poten-
tially will be immunogenic in humans. Also, because it

15   contains murine constant sequences, it will likely not
exhibit the full range of human effector functions and will
probably be more rapidly cleared if administered to humans.
While such disadvantages should not be problematic in the
treatment of some disease conditions or persons, they pose

20   substantial concern if the disease treated is of a chronic
or recurrent nature. Examples of recurrent or chronic
diseases include, e.g., autoimmune diseases, wherein the
host continually or chronically exhibits an autoimmune
reaction against self-antigens.

25   Therefore, in order to alleviate the disadvantages
associated with murine antibody 24-31, namely potential
immunogenicity in humans and decrease of human effector
functions, the present inventors desired to produce im-
proved, humanized derivatives of the murine 24-31 antibody.

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While this was the goal of the present invention, the desired result was not of a routine or predictable nature. Humanization of antibodies requires the careful selection of amino acid residues which are to be modified, and the

5   judicious selection of residues which are to be substituted therefor. This is because modification of antibody variable regions, even those involving a few amino acid residues, may cause substantial deleterious effects on antigen binding. For example, humanized antibodies may exhibit

10 substantially reduced antigen affinity and/or antigen-specificity in relation to the parent antibody.

As noted *supra*, different methods of humanization of antibodies, including murine antibodies have been reported in the literature. See, e.g., Padlan, *Molec. Immunol.*,

15 31(3):169-217 (1994); Padlan, *Molec. Immunol.*, 28:484-498 (1991); Morrison and Oi, *Adv. Immunol.*, 44:65-92 (1988), all of which references are incorporated by reference in their entirety herein. These methods include in particular humanization by CDR grafting (Jones et al, *Nature*, 321:522-

20 525 (1986); Verhoeyen et al, *Science*, 239:1534-1539 (1988); and the more tailored approach of Padlan, *Molec. Immunol.*, 28:489 (1991) and Padlan, *Molec. Immunol.*, 31:169 (1994) which involves the selection of non-essential framework amino acid residues and their modification by appropriate

25 substitution mutation. These references are incorporated by reference in their entirety herein.

As noted, CDR grafting techniques, while successful in some instances, may substantially adversely affect the affinity of the resultant humanized antibodies. This is

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believed to occur because some framework residues affect or
are essential for and at least affect antigen binding. Our
technique; Padlan (1994) (*Id.*)) is more refined because we
retain only those murine framework residues which we deem
5   critical to the preservation of the antibody combining site
while keeping the surface properties of the molecule as
human as possible. Accordingly, this technique has the
potential of producing humanized antibodies which retain
the antigen-binding characteristics of the parent antibody.
10   Because of this, this technique was selected by the present
inventors as the means by which humanized antibodies de-
rived from murine antibody 24-31 specific to human gp39
would potentially be obtained.

The cloning of the variable regions of 24-31
15   (described in detail in the examples *infra*) resulted in the
identification of the V_L and V_H sequences utilized by the
24-31 antibody respectively shown in **Figure** 7 and **Figure** 8.
After sequencing, the variable regions were then humanized.
As noted, this was effected substantially according to the
20   method of Padlan (1994) (*Id.*), incorporated by reference
supra.

This method generally comprises replacement of the
non-human framework by human framework residues, while
retaining only those framework residues that we deem criti-
25   cal to the preservation of antigen binding properties.
Ideally, this methodology will confer a human-like charac-
ter on the surface of the xenogeneic antibody thus render-
ing it less immunogenic while retaining the interior and

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contacting residues which affect its antigen-binding properties.

More specifically, the 24-31 V_K and V_H sequences set forth in **Figures 7** and **8** were humanized by comparison to
5   human antibodies of reported sequence, which are referred to as "templates."

Specifically, the 24-31 V_K was humanized using as templates:

(a) For VL#1, the human V-Kappa subgroup I sequences,
10   e.g., DEN and the like, as well as the germline 012 (see Cox et al, *Eur. J. Immunol.* 24:827-836 (1994)), and for VL#2, the human V-Kappa subgroup IV sequences, e.g., LEN. Such template sequences are known and are reported in Kabat et al (1991) (*Id.*) or GenBank.

15   The 24-31 V_H #1 was humanized using as templates

(a) the human V_H subgroup IV sequence, 58p2 and

(b) (GenBank Accession No.) Z18320 and the germline 3d75d (S. van der Maarel et al, *J. Immunol.*, 150:2858-2868 (1993).

20   Such template variable heavy antibody sequences are also known and are reported in Kabat et al, "Sequences of Proteins of Immunological Interest," 5th Ed., NIH (1991) and in GenBank.

The template human variable heavy and light sequences
25   were selected based on a number of different criteria, including, in particular, high degree of sequence similarity with 24-31 overall, as well as similarity in the "important" residues, i.e., those which are believed to be comprised in the V_L:V_H interface; those which are in contact

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with the complementarity determining regions, or which are inwardly pointing. Also, the templates were selected so as to potentially preserve the electrostatic charge of the 24-31 F_v as much as possible, and also so as to preserve

5   glycines, prolines and other specific amino acid residues which are believed to affect antigen binding.

This methodology resulted in the following preferred humanized V_L and V_H heavy sequences derived from the 24-31 antibody which are set forth below in **Table 1** and **Table 2**.

10   As discussed above, the invention further embraces equivalents and variants of these preferred humanized sequences, e.g., those containing one or more conservative amino acid substitutions which do not substantially affect gp39 binding. The complementarity determining regions are identi-

15   fied in **Figures** 7 and **8** which contain the entire variable heavy and light chain CDR sequences of the parent (non-humanized) 24-31 antibody.

TABLE 1

HUMANIZED 24-31 VL SEQUENCES

```
                10        20              40              60          70        80

24-31  DIVMTQSQKFMSTSVGDRVSITC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT GVPDRFSGSGSGTDFTLTISNMQSEDLADYFC QQYNSYPYT
       100
       FGGGTKLEIK

(1)    DIVMTQSPSFLSASVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT GVPDRFSGSGSGTDFTLTISSLQPEDFADYFC QQYNSYPYT
       FGGGTKLEIK

(2)    DIVMTQSPDSLAVSLGERATINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT GVPDRFSGSGSGTDFTLTISSLQAEDVADYFC QQYNSYPYT
       FGGGTKLEIK

(3)    DIVMTQSPSFMSTSVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRT  GVPDRFSGSGSGTDFTLTISSMQPEDFADYFC QQYNSYPYT
       FGGGTKLEIK

(4)    DIVMTQSPDSMATSLGERVTINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT GVPDRFSGSGSGTDFTLTISSMQAEDVADYFC QQYNSYPYT
       FGGGTKLEIK
```

TABLE 2

HUMANIZED 24-31 VH SEQUENCES

```
                10        20        30        40                                              70        82abc     90
24-31  EVQLQESGPGLVKPSQTLSLTCSVTGDSIT NGFWI WIRKFPGNKLEYMG YISYSGSTYYNPSLKS RISITRDTSQNQFYLQLNSVTTEDTGTYYCAC
              110
       RSYGRTPYYFDF WGQGTTLTVSS

(1)    EVQLQESGPGLVKPSETLSLTCTVSGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS RISISRDTSKNQFSLKLSSVTAADTGVYYCAC
       RSYGRTPYYFDF WGQGTTLTVSS

(2)    EVQLQESGPGLVKPSQTLSLTCTVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS RISISRDTSKNQFSLKLSSVTAADTGVYYCAC
       RSYGRTPYYFDF WGQGTTLTVSS

(3)    EVQLQESGPGLVKPSQTLSLTCAVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS RISISRDTSNNQFSLNLNSVTRADTGVYYCAC
       RSYGRTPYYFDF WGQGTTLTVSS

(4)    EVQLQESGPGLVKPSETLSLTCAVYGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS RISISRDTSKNQFYLKLSSVTAADTGVYYCAC
       RSYGRTPYYFDF WGQGTTLTVSS
```

5

10

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As can be seen therefrom, four preferred humanized framework sequences were designed for both the V_H and V_L chains. Therefore, there are 16 different possible humanized 24-31 antibodies which may be synthesized using the

5   above-identified humanized V_H and V_L 24-31 sequences, excluding variants and equivalents containing conservative modifications.

Humanized 24-31 antibodies containing these humanized variable heavy and light sequences may be obtained by

10   recombinant methods. It is expected that humanized sequences which contain any combination of the above preferred humanized variable sequences will result in humanized antibodies which bind human gp39. Moreover, based on these sequences, the order of preference using the number-

15   ing set forth in **Table 1** and **Table 2** is expected to be as follows:

(1)   #1 V_L with #1 V_H (Version 1)

(2)   #2 V_L with #1 V_H (Version 2)

(3)   #1 V_L with #2 V_H (Version 3)

20   (4)   #2 V_L with #2 V_H (Version 4)

The above-identified humanized V_H and V_L sequences may be further modified, e.g., by the introduction of one or more additional substitution modifications and also by the addition of other amino acids. Additional modifications

25   will be selected which do not adversely affect antigen (gp39) binding. For example, the inventors contemplate further modification of the V_H chain by substitution of one or more of residues 34, 43, 44 and 68 (according to Kabat numbering scheme) Kabat et al (1991) (*Id.*). Also, the

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inventors contemplate modification of residue 85 of the V_L chain. Based on the structural features of the antibody combining site, it is believed that modification of such residues should also not adversely impact antigen binding.

5   Moreover, it is expected that the introduction of one or more conservative amino acid substitutions should not adversely affect gp39 binding.

So as to better describe the subject humanized 24-31, V_H and V_L sequences, the preferred humanized framework

10   sequences are also set forth in **Table 3** below, which compares these sequences to the template human variable heavy and light framework sequences, i.e., human DEN VK1, Human o12/V36 germline, human LEN VKIV, human 58p2, human Z18320, and human 3d75d as well as to the unhumanized murine 24-31

15   V_H and V_L framework sequences.

TABLE 3

VK Framework Region Comparisons - Humanized Anti-gp39

	FR1	FR2
Human 012/V3b germline	DIQMTQSPSFLSASVGDRVTITC	WYQQKPGKAPKLLIY
Human DEN VKI	----------T--------------	-------E---V---
Murine 24-31	--V----QK-M-T------S---	-------QS------
Padlan VL#1 humanized	--V--------------------	---------S------

	FR3	FR4
Human 012/V3b	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	
Human DEN VK1	--------------E---------SD-------	FGQGTKLEIK
Murine 24-31	---D----------------NM-SE-L-D-F-	--G-------
Padlan VL#1	---D-----------------------D-F-	--G-------

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TABLE 3 (Continued)

                              FR1   FR2

    Human LEN VKIV   DIVMTQSPDSLAVSLGERATINC   WYQQKPGQPPKLLIY

    Murine 24-31   -------QKFMST-V-D-VS-T-   --------S------

5   Padlan VL#2 humanized ------------------------   --------S------

                    FR3   FR4

    Human LEN VKIV   GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC   FGQGTKLEIK

    Murine 24-31   --------------------NM-S--L-D-F-   --G-------

    Padlan VL#2   ----------------------------D-F-   --G-------

10   FR1

    Human 58p2   QVQLQESGPGLVKPSETLSLTCTVSGGSIS

    Murine 24-31   E---------S----Q------S-T-D--T

    Padlan VH#1 humanized E------------------------D--T

    Human Z18320 GenBank ---------------Q--------------

15 Human 3d75d germline ---------------Q--------------

    Padlan VH#2 humanized E--------------Q----------D--T

                    FR2   FR3

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```
      Human 58p2       WIRQPPGKGLEWIG    RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR

      Murine 24-31     ---KF--NK--YM-    -IS-TR---Q---Y-Q-N---TE--GT----C

      Padlan VH#1      ---K---NK--YM-    -IS--R-------------------G-----C


      Human Z18320     -----A--------    --------------------------------

 5    Human 3d75d      ----H--------     --------------------------------

      Padlan VH#2      ---KH--NK--YM-    -IS--R-------------------G-----C




      Human 58p2       WGQGTMVTVSS

      Murine 24-31     -----TL----

      Padlan VH#1      -----TL----


10    Human Z18320     -----------

      Padlan VH#2      -----TL----
```


In order to produce humanized antibodies, DNA sequenc-
es are synthesized which encode for the afore-identified
humanized V_L and V_H sequences. As noted, taking into ac-
15   count these four humanized V_L sequences, and four humanized
V_H sequences, there are 16 potential humanized antigen
combining sites which may be synthesized. Also, there are
even more potential humanized antigen combining sites
taking into account the potential substitution of residues
20   34, 43, 44 and 68 of the humanized V_H and residue 85 of the
humanized V_L by other amino acid residues and/or the poten-
tial incorporation of conservative substitution mutations.
Two of the preferred humanized variable light sequences (1)
and (2) and a preferred humanized variable heavy sequence

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(1) including the complementarity determining regions and corresponding DNA sequences are set forth in **Figures 4, 5** and **6**, respectively.

Methods for synthesizing DNA encoding for a protein of known sequence are well known in the art. Using such methods, DNA sequences which encode the subject humanized V_L and V_H sequences are synthesized, and then expressed in vector systems suitable for expression of recombinant antibodies. This may be effected in any vector system which provides for the subject humanized V_L and V_H sequences to be expressed as a fusion protein with human constant domain sequences and associate to produce functional (antigen binding) antibodies.

Expression vectors and host cells suitable for expression of recombinant antibodies and humanized antibodies in particular, are well known in the art.

The following references are representative of methods and vectors suitable for expression of recombinant immunoglobulins which are incorporated by reference herein: Weidle et al, *Gene*, 51:21-29 (1987); Dorai et al, *J. Immunol.*, 13(12):4232-4241 (1987); De Waele et al, *Eur. J. Biochem.*, 176:287-295 (1988); Colcher et al, *Cancer Res.*, 49:1738-1745 (1989); Wood et al, *J. Immunol.*, 145(a):3011-3016 (1990); Bulens et al, *Eur. J. Biochem.*, 195:235-242 (1991); Beggington et al, *Biol. Technology*, 10:169 (1992); King et al, *Biochem. J.*, 281:317-323 (1992); Page et al, *Biol. Technology*, 9:64 (1991); King et al, *Biochem. J.*, 290:723-729 (1993); Chaudary et al, *Nature*, 339:394-397 (1989); Jones et al, *Nature*, 321:522-525 (1986); Morrison

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and Oi, *Adv. Immunol.*, 44:65-92 (1988); Benhar et al, *Proc. Natl. Acad. Sci. USA*, 91:12051-12055 (1994); Singer et al, *J. Immunol.*, 150:2844-2857 (1993); Cooto et al, *Hybridoma*, 13(3):215-219 (1994); Queen et al, *Proc. Natl. Acad. Sci.*

5   *USA*, 86:10029-10033 (1989); Caron et al, *Cancer Res.*, 32:6761-6767 (1992); Cotoma et al, *J. Immunol. Meth.*, 152:89-109 (1992). Moreover, vectors suitable for expression of recombinant antibodies are commercially available.

        Host cells known to be capable of expressing function-
10   al immunoglobulins include by way of example mammalian cells such as Chinese Hamster Ovary (CHO) cells, COS cells, myeloma cells, bacteria such as *Escherichia coli*, yeast cells such as *Saccharomyces cerevisiae*, among other host cells. Of these, CHO cells are used by many researchers
15   given their ability to effectively express and secrete immunoglobulins.

        Essentially, recombinant expression of humanized antibodies are effected by one of two general methods. In the first method, the host cells are transfected with a
20   single vector which provides for the expression of both heavy and light variable sequences fused to selected constant regions. In the second method, host cells are transfected with two vectors, which respectively provide for expression of either the variable heavy or light sequence
25   fused to selected constant regions.

        Human constant domain sequences are well known in the art, and have been reported in the literature. Preferred human V_L sequences includes the Kappa and lambda constant light sequences. Preferred human heavy constant sequences

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include human gamma 1, human gamma 2, human gamma 3, human gamma 4 and mutated versions thereof which provide for altered effect or function, e.g. enhanced *in vivo* half-life and reduced Fc receptor binding.

5   Preferred modifications of the human gamma 4 constant domain include P and/or E modifications, which respectively refer to the change of a leucine to a glutamic acid at position 236 and/or the change of a serine to a proline (Kabat numbering) at position 229 such as described in 10   commonly assigned Attorney Docket No. 012712-165 filed on September 6, 1995 and incorporated by reference in its entirety herein.

A particularly preferred vector system comprises the expression vectors described in commonly assigned U.S. 15   Serial No. 08/476,237 filed June 7, 1995, Serial No. 08/397,072, filed January 25, 1995 and 07/912,122 filed July 10, 1992, 07/886,281 filed March 23, 1992, and 07/735,064 filed July 25, 1991, all incorporated by reference in their entirety.

20   In particular, these applications describe vector systems for the production of recombinant antibodies, referred to as TCAE 5.2 and TCAE 6 which comprise the following:

1)   Four transcriptional cassettes in tandem order:

25   (a)   a human immunoglobulin light chain constant region. In TCAE 5.2 this is the human immunoglobulin Kappa light chain constant region (Kabat numbering amino acids 108-214, allotype Km 3) and in TCAE 6 the human immunoglobulin light chain lambda constant region (Kabat numbering

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amino acids 108-215, genotype Oz minus, Mcg minus, Ke minus allotype).

(b)   a human immunoglobulin heavy chain constant region; in both constructs the human immunoglobulin heavy chain is a gamma/constant region (Kabat numbering amino acids 114-478 allotype Gmla, Gm12).

(c)   DHFR; containing its own eukaryotic promoter and polyadenylation region; and

(d)   NEO; also containing its own eukaryotic promoter and polyadenylation region.

2)   The human immunoglobulin light and heavy chain cassettes contain synthetic signal sequences for secretion of the immunoglobulin chains; and

3)   The human immunoglobulin light and heavy chain cassettes contain specific DNA links which allow for the insertion of light and heavy immunoglobulin variable regions which maintain the translational reading frame and do not alter the amino acids normally found in immunoglobulin chains.

These vectors are preferably utilized in CHO cells. The subject antibodies are preferably expressed in the above-described vector systems.

However, the subject humanized antibody sequences derived from the number 24-31 antibody may be expressed in any vector system which provides for the expression of functional antibodies, i.e., those which bind gp39 antigen.

In particular, the inventors elected to express the subject humanized V_L and V_H sequences, as well as the native (unmodified) V_L and V_H sequences derived from 24-31 in CHO

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cells using the N5KG1 expression vector which contains
human Kappa and human gamma 1 constant regions. The N5KG1
expression vector is depicted schematically in **Figure 1**.
As hoped, the chimeric antibody derived from 24-31, when
5   expressed in CHO cells binds gp39 (by demonstrated binding
to CHO- gp39 transfectant). Also, several humanized anti-
bodies of the invention derived from 24-31 when expressed
using this vector system resulted in functional (gp39
binding) antibodies.

10   The present invention is further described through
presentation of the following examples. These examples are
offered by way of illustration and not by way of limita-
tion.

EXAMPLE 1

15   <u>**Selection of 24-31 Antibody for Humanization**</u>.

Accumulating evidence in animal models indicates that
anti-gp39 administration prevents a variety of autoimmune
processes and interferes with allograft rejection. These
results provide compelling evidence that antibodies to
20   human gp39 may have significant therapeutic value in the
management of autoimmune disease and the transplantation of
allogeneic tissue and organs in humans. A monoclonal
antibody (mAb) specific for human gp39 has been reported
(Lederman et al, *J. Immunol.*, 199:3817 (1992)), and its
25   functional activity in blocking gp39-CD40 interactions *in
vitro* has been evaluated. To gain greater insights into
the functional impact of anti-gp39 antibodies on the human

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immune system, a panel of anti-human gp39 mAbs was generated. From this panel, one mAb appeared superior and was extensively tested for functional inactivation of gp39 *in vitro* and *in vivo*.

5   More specifically, a panel of 6 murine (all IgG1) anti-gp39 antibodies was generated by immunization with a soluble fusion protein of human gp39 (gp39-CD8), followed by challenge with activated human peripheral blood T cells. Flow cytometric analysis of human peripheral blood T cells

10   demonstrated that the mAbs recognized a cell surface molecule expressed on activated (PMA/ionomycin), but not resting, CD3$^+$ T cells, and that the pattern of reactivity was similar to that seen with a recombinant CD40 fusion protein (CD40-Ig) (data not shown). Immunoprecipitation of [^{35}S]

15   metabolically labeled activated human peripheral blood T cells revealed that each of the 6 mAbs precipitated a molecule of similar size (33 kDa) to that precipitated by CD40-Ig. Finally, binding of CD40-Ig to gp39 was blocked in the presence of the antibodies indicating recognition of

20   the same molecule, further confirming their specificity. Although all 6 mAbs were capable of blocking gp39 function, one mAb, 24-31, was selected for extensive analysis.

EXAMPLE 2

T cell-dependent B cell proliferation and

25   **differentiation (Ig production) is blocked by anti-gp39**.

A number of studies have provided evidence that signals delivered through CD40 by its ligand, gp39, induce B cell activation, proliferation, differentiation, and isotype switching. To determine if the anti-gp39 24-31 mAb

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blocked gp39 function, B cells were cultured with a soluble
fusion protein of gp39 (gp39-CD8) in the presence or ab-
sence of 24-31, and the B cell proliferative response was
assessed by ^{3}H-thymidine incorporation. The results, shown

5   in **Figure 2A**, demonstrate that gp39-CD8 induced vigorous
proliferation of B cells. The presence of anti-gp39 24-31
mAb completely ablated B cell proliferation induced by
gp39-CD8 at concentrations as low as 2.5 μg/ml. To deter-
mine whether 24-31 interfered with T cell-induced B cell

10   differentiation, B cells were co-cultured with anti-CD3
activated T cells in the presence or absence of 24-31.
Polyclonal IgM, IgG, and IgA production was assessed after
12 days. As shown in **Figure 2B**, the addition of 24-31
inhibited polyclonal IgM, IgG, and IgA antibody production

15   (90-99%). These results confirm previous reports estab-
lishing the requirement for gp39-CD40 interactions in T
cell-dependent B cell differentiation (Nishioka et al, *J.
Immunol.*, 153:1027 (1994), and further demonstrate the use
of newly characterized anti-human gp39 24-31 mAb in block-

20   ing gp39 function.

EXAMPLE 3

**Anti-gp39 blocks *in vivo* tetanus toxoid specific antibody
production in *SCID* mice reconstituted with human PBL**.

Numerous studies have established that the human

25   immune system can be studied *in vivo* under experimental
conditions through the use of severe combined immunodefi-
ciency (*scid*) mice engrafted with human peripheral blood
lymphocytes (hu-PBL-*scid* mice) (Mosler et al, *Nature*,

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335:256 (1988); McCune et al, *Science*, 241:1632 (1988).
Long-term chimerism is achieved in *scid* mice by injection
with human PBL, and antigen-specific secondary antibody
responses are detected in hu-PBL-*scid* mice challenged *in*

5   *vivo* with antigen (Carlsson et al, *J. Immunol.*, 148:1065
(1992); Duchosal et al, *Cell Immunol.*, 139:468 (1992)).
This system was exploited to evaluate the immunosuppressive
effects of *in vivo* anti-gp39 administration on the immune
responses elicited by human T and B cells.

10   Experiments, the results of which are contained in
Figure 2B, demonstrated that blockade of gp39 function by
24-31 inhibited T cell-dependent polyclonal Ig production
by human B cells *in vitro*. To determine whether 24-31
could also inhibit antigen specific B cell antibody produc-

15   tion *in vivo*, C.B-17 *scid/scid* mice injected i.p. with
human PBL (hu-PBL-*scid*) and immunized with tetanus toxoid
(TT) were treated with 24-31 or PBS, and the secondary
(IgG) anti-TT antibody response was assessed. Immunization
of hu-PBL-*scid* with TT resulted in detectable levels of IgG

20   anti-TT antibody within 14 days post immunization in most
animals (**Table 4**). However, treatment with anti-gp39 (24-
31; 250 μg/day, twice weekly) completely ablated the sec-
ondary anti-TT antibody response in 9/10 mice examined,
demonstrating that *in vivo* blockade of gp39 function also

25   resulted in inhibition of antigen specific humoral
responses.

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Recipient Strain*	Treatment¶	Anti-Tetanus Antibody (O.D. ± SE)§ (Frequency of Mice Containing Anti-Tetanus Antibody)			
		days post immunization			
		7d	14d	21d	28d
C.B-17 scid/scid	PBS	<0.02 (0/10)	2.30 ± .042 (7/10)*	.224 ± .040 (8/10)**	.137 + .007 (4/10)
	anti-gp39	.162 (1/10)	<0.02 (0/10)	<0.02 (0/10)	<0.02 (0/10)

Table 4. Ablation of the secondary anti-tetanus antibody response following engraftment of human PBL in C.B-17 *scid/scid* mice immunized with tetanus toxoid.* Four-six week old C.B-17-*scid/scid* mice were injected i.p. with 20 x 10⁶ human PBL and 0.25 ml tetanus toxoid.

¶Anti-gp39 24-31 or PBS (250 µg/injection) was administered i.p. twice weekly throughout the entire experiment.

§The level of human anti-tetanus toxoid antibody in the serum was determined weekly by ELISA. All mice with serum levels of human anti-tetanus toxoid antibody > 0.100 O.D. at a 1:10 dilution were considered positive. Only positive mice were used in the calculation of the mean ± SE values included in the table. The level of human anti-tetanus toxoid in sera from pre-immune mice not immunized with tetanus toxoid was < 0.02 O.D. Data are presented as mean ± SE.

 *Significantly different (p=0.222) than the anti-gp39 treated group.

**Significantly different (p<0.001) than the anti-gp39 treated group.

EXAMPLE 4

Anti-gp39 treatment does not inhibit the antigen-specific T cell proliferative response of hu-PBL-*scid* spleen cells.

To determine whether treatment of hu-PBL-*scid* mice with anti-gp39 altered the responsiveness of antigen-specific T cells *in vivo*, the proliferative response of spleen cells from hu-PBL-*scid* mice immunized with TT and treated with 24-31 was assessed *in vitro*. Spleen cells from control or anti-gp39 treated hu-PBL-*scid* mice were cultured with TT or medium alone, and the proliferative response was

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assessed by ^{3}H-thymidine incorporation after 6 days. **Table 5** summarizes the results of one such experiment. Hu-PBL-*scid* mice treated with anti-gp39 responded similarly to *in vitro* stimulation with TT as did hu-PBL-*scid* mice which

5   were untreated (5/10 vs. 3/10 responding mice). Experiments using NOD/LtSz-*scid*/*scid* mice as recipients yielded similar results, although anti-TT antibodies were undetectable in these mice (data not shown). These data demonstrate that treatment with anti-gp39 does not result in

10   deletion or functional inactivation of antigen-specific T cells in hu-PBL-*scid* mice and support the contention that inhibition of TT specific antibody responses by anti-gp39 is due to blockade of gp39-CD40 interactions and subsequent B cell responses rather than T cell inactivation.

	Recipient Strain*	Treatment¶	Frequency of Responding Mice§
	C.B-17 *scid*/*scid*	PBS	3/10
		anti-gp39	5/10
	NOD/LtSz-*scid*/*scid*	PBS	5/10
		anti-gp39	6/10

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Table 5. Anti-gp39 treatment does not alter the anti-
tetanus T cell proliferative response following
engraftment of human PBL in C.B-17-*scid/scid* or NOD/LtSz-
scid/scid mice immunized with tetanus toxoid.

5   *Four-six week old C.B-17-*scid/scid* or NOD/LtSz-*scid/scid*
mice were injected i.p. with 20 x 10^6 human PBL and 0.25
ml tetanus toxoid.

¶Anti-gp39 24-31 or PBS (250 μg/injection) was adminis-
tered i.p. twice weekly throughout the entire experiment.
10 §Spleen cells from mice injected with human PBL and immu-
nized with tetanus toxoid were cultured at a concentration
of 1 x 10^5 cells/ml in the presence of media alone or
tetanus toxoid (2.5 or 5.0 μg/ml). Proliferation was
assessed by ^{3}H-thymidine incorporation after 6d. Stimula-
15 tion indices were calculated by the following formula"
S.I. = cpm tetanus - cpm medium/cpm medium. S.I. of >
than 2.0 was considered positive.

EXAMPLE 5

Generation of a gp39 CHO transfectant cell line.

20   Recently, a CHO transfectant that constitutively
expresses cell-surface gp39 was generated to use as a
reagent for the humanized anti-gp39 24-31 binding studies
proposed in this application. The full-length gp39 gene
(Hollenbaugh et al, *Immunol. Rev.*, 138:23 (1994)) was
25 amplified by polymerase chain reaction (PCR) of phytohemag-

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glutinin-activated human PBL and cloned into IDEC's INPEP4
vector under the transcriptional control of the cytomegalo-
virus (CMV) promoter and enhancer elements. A CHO trans-
fectant was established and amplified in 50 nM methotrex-
5   ate. The transfectant, 50D4, was shown to express cell-
surface gp39 by ELISA (data not shown) and FACS analysis
(Figure 3).

EXAMPLE 6

High-level expression of antibodies

10   **using a CHO expression system.**

IDEC's proprietary N5KG1 expression vector is used in
CHO cells for expression of the humanized anti-gp39 24-31
antibody. This vector is depicted schematically in **Fig-
ure 1**. High-level expression of recombinant antibodies is
15   consistently obtained in CHO cells using this vector and
similar vectors. Using these vectors, a high percentage of
G418 resistant clones, 5-10%, are found to express signifi-
cant amounts of recombinant proteins (1-10 mg/l of anti-
body). These are usually single plasmid copy integrants,
20   and can easily be amplified using methotrexate to obtain
30-100 pg/cell/day of secreted immunoglobulin. **Table 6**
lists the antibody levels obtained before and after gene
amplification of 3 antibodies expressed in CHO cells uti-
lizing this system.

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Antibody	before amplification mg/l	after amplification in spinner flask mg/l	after amplification in fermentor mg/l
Anti-CD4 γ1	1-2	100-110	950
Anti-CD4 γ4	3-4	125-150	N.D.
Anti-CD20	5-10	200-300	650

5   **Table 6. Antibody production levels using IDEC's CHO expression technology.**

EXAMPLE 7

Cloning of 24-31 V_k and V_H DNA Sequences

The anti-gp39 24-31 V_k and V_H gene segments were cloned
and sequenced. Following analyses of their sequences,

10   humanized versions of the V region gene segments were
designed. The corresponding DNA sequences were synthesized
and cloned into a high-level expression vector containing
human constant region genes. A CHO transfectant producing
the humanized 24-31 antibody is then established. To

15   confirm that the humanized version of the anti-gp39 anti-
body retains its gp39 binding affinity, the relative affin-
ities of the murine and humanized antibodies were compared
in direct binding and competition assays. In addition, the
ability of the humanized 24-31 to block CD40 binding to

20   gp39 and to inhibit T cell-dependent antibody production is
evaluated.

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1.   Cloning of the 24-31 V_k and V_H gene segments

a.   Preparation of cDNA.   PolyA⁺ mRNA was pre-
pared from 2 x 10^6 cells each of the 24-31 hybridoma and the
NS1 cell line, (Carroll et al, *Mol. Immunol.*, 10:991

5   (1988)), the fusion partner used in the generation of the
24-31 hybridoma, utilizing an Invitrogen Corporation Micro-
Fast Track™ mRNA isolation kit, according to the manufac-
turer's protocol.   First strand cDNA was synthesized uti-
lizing 50 pmoles oligo-dT and 5 units M-MLV reverse tran-

10   scriptase (Promega) (Sambrook et al, *Molecular Cloning: A
Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory
Press (1989)) followed by Sephadex G-25 chromatography.

b.   PCR amplification Of V_k and V_H cDNA. 24-31
and NS1 cDNA were amplified by PCR using a panel of 5'

15   primers specific for V_k or V_H leader sequences in combina-
tion with 3' constant region primers.   The panel of 5'V_H
primers are identical to those described by Jones and
Bendig (*Bio/Technol.*, 9:88 (1991); Errata, *Bio/Technol.*,
9:579 (1991)).   The panel of 5'V_k primers (Jones et al,

20   (*Id.*)) were modified to convert the Sal I cloning site
recognition sequences (GTCGAC) into Bgl II recognition
sequences (AGATCT) to facilitate the cloning of the ampli-
fied gene segments into IDEC's N5KG1 expression vector
(See **Figure 1**).   The 3' V_k and V_H primers contain a Bsi WI

25   cloning site sequence at amino acid positions 108-109
(numbering according to Kabat et al, "Sequences of Proteins
of Immunological Interest," 5th Ed., NIH (1991)) and a Nhe
I cloning site sequence at positions 114-115, respectively,
and have the following sequences:

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TGCAGCATC<u>CGTACG</u>TTTGATTCCAGCTT(C_k) and

GGGGGTGTCGT<u>GCTAGC</u>TG(A/C)(G/A)GAGAC(G/A)GTGA (Cγ1). This
primer panel has been previously used by the Assignee to
amplify and clone the C2B8 anti-CD20 antibody (Nishioka et

5   al, *J. Immunol.*, 153:1027 (1994)) and numerous other mouse
V_k and V_H gene segments (data not shown).

In order to determine the correct primer pair for the
amplification of the 24-31 V_k and V_H gene segments, the 24-
31 cDNA were amplified in 23 individual reactions contain-

10   ing one of the 11 5'V_k primers in combination with the C_K
primer or one of the 12 5'V_H primers in combination with the
Cγ1 primer. For comparison, NS1 cDNA was amplified using
the same panel of primers. 1μl cDNA (1/50 of the cDNA
sample) was amplified in a 100 μl final volume containing 5

15   units *Taq* DNA polymerase (Perkin Elmer), 10 mM Tris-HCl, pH
8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM each of dCTP, dGTP,
dATP, and TTP, 50 pmoles 3' constant region primer, and 50
pmoles 5' primer. The amplification cycle consisted of
denaturation for 1 minute at 95°C, annealing for 2 minutes

20   at 50°C, and extension for 2 minutes at 72°C, repeated 34
times. The amplified products were analyzed by agarose gel
electrophoresis. The 24-31 PCR reactions yielding a unique
amplified product for V_k and for V_H were repeated and the
products from duplicate PCR reactions cloned. PCR ampli-

25   fied products are agarose gel-purified (Sambrook et al,
Molecular Cloning: A Laboratory Manual, 2nd Ed. (1989)) and
digested with Bgl II and Bsi WI (for V_k) or Sal I and Nhe I
(for V_H). The products are ligated (Ausabel et al, *Current*

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Protocols in Molecular Biology, Vol. 2, Greene Publ. Assoc. (1992)) sequentially into IDEC's vector, N5KG1.

Following transformation of *E. coli* XL1-blue cells (Stratagene), plasmid DNA was prepared, and the V_k and V_H

5   sequences obtained from the duplicate constructs (sequencing performed by Scripps Research Institute Core Facility, La Jolla, CA). The sequences of the endogenous light and heavy chains of the NS1 fusion partner are known (Carroll et al, *Mol. Immunol.*, 10:991 (1988); Kabat et al, (1991)

10   (*Id.*)) and were used to distinguish PCR products resulting from the amplification of the 24-31 versus the NS1 fusion partner V regions.

EXAMPLE 8

Synthesis of gene segments encoding humanized 24-31 V

15   **regions.**

Humanized versions containing the most preferred humanized 24-31 V_k and V_H sequences identified in **Tables 1** and **2** as humanized V_L and V_H (1) were synthesized. Specifically, four pairs of overlapping, complementary olionucleo-

20   tides (oligos) encoding the above-identified humanized V_k or V_H regions were synthesized (Midland Chemicals) and purified by denaturing polyacrylamide gel electrophoresis (Ausubel et al, *Current Protocols in Molecular Biology*, Vol. 2, Greene Publ. Assoc. (1992)). Each oligo is approximately

25   100 bases in length and overlap by 20 bases the adjacent complementary oligonucleotide. The V_k and V_H 5' oligos contain Bgl II and Sal I cloning sites and the 3' oligos possess Bsi WI and Nhe I cloning sites, respectively. Each variable region gene segment was assembled from the syn-

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thetic oligos, diagrammed below, using the following proce-
dure (summarized in Watson et al, *Recombinant DNA*, 2nd Ed.,
Scientif. Amer. Books, NY, NY (1992)). Complementary oligo
pairs (A+E, B+F, C+G, D+F) were kinased using 300 pmoles of

5   each primer and T4 polynucleotide kinase (Promega) accord-
ing to the manufacturer's protocol. The oligos were an-
nealed by heating to 95°C and slow cooling to room tempera-
ture. The annealed oligo pairs were ligated (A/E with B/F
and C/G with D/H) utilizing 6 units T4 DNA ligase (New

10   England Biolabs). After digestion with the appropriate 5'
or 3' cloning site restriction endonuclease, the approxi-
mately 200 base pair DNA fragments were purified by elec-
troelution following polyacrylamide gel electrophoresis
(Sambrook et al, (*Id.*)). The synthetic gene fragments were

15   then inserted into IDEC's proprietary high-level expression
vector, N5KG1, under the transcriptional control of the CMV
promoter and enhancer elements. The ligation reaction
contains the 2 gel-purified fragments (A/E/B/F and C/G/D/H)
and N5KG1 at a molar ratio of 100:100:1, respectively.

20   After transformation of XL1-blue cells, plasmid DNA was
prepared and the sequences of the synthetic gene segments
confirmed. The resulting construct, h24-31, encodes the
humanized 24-31 V region segments and human kappa and gamma
1 constant regions. As indicated, this antibody contains

25   the humanized variable heavy and humanized variable light
sequences identified in **Table 1** and **Table 2** as the "(1)"
sequences, which are predicted to provide for humanized
antibody having optimal gp39 properties. In addition, a
construct was generated which contains V_L#2 in combination

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with V_H#1 (version 2 of humanized 24-31). Similar con-
structs utilizing IDEC's proprietary vectors have been used
for high-level expression of IDEC's anti-CD20 (Reff et al,
Blood, 83:425 (1994)) and anti-CD4 (Newman et al, *Biol.*
Technology, 10:1455 (1992)) antibodies.

```
            A           B           C           D
5 ' _____  _____  _____  _____  3'

    _____  _____  _____  _____
            E           F           G           H
```

EXAMPLE 9

2. Production and characterization of humanized 24-31

a. Generation of CHO transfectants producing humanized 24-31 (version 1 and version 2).

CHO transfectants expressing humanized 24-31 (version
1 or version 2) were generated by electroporation of 4 x 10^6
CHO cells with linearized h24-31 DNA (version 1 or version
2) followed by selection in G418. The cell culture super-
natants from G418 resistant clones were assayed for immuno-
globulin production by sandwich ELISA employing a goat
anti-human kappa to capture the immunoglobulin. Immuno-
globulin binding was measured by incubating with a horse
radish peroxidase (HRP)-conjugated goat antibody specific
for human IgG, followed by HRP substrate, 0.4 mg/ml O-
Phenylene-diamine (OPD) in a citrate buffer (9-34 g/l $C_6H_8O_7$
and 14.2 g/l Na_2HPO_4), pH 5.0, including 0.0175% H_2O_2. The
plate was read in a Molecular DeviCes "Vmax, kinetic micro-
plate reader" spectrophotometer at 490 nm.

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EXAMPLE 10

b.   **Characterization of humanized 24-31 (version 1).**

The humanized anti-gp39 24-31 antibody is evaluated
initially for direct binding to cell surface gp39 expressed
5   on 50D4, the gp39 CHO transfectant described in **Example 5**.
Supernatants from the G418-resistant h24-31 CHO transfect-
ants that produce immunoglobulin are tested for binding to
50D4 cells and, as negative control, to CHO cells. In this
assay 50D4, 1 x 10^5 /well, are bound to the bottom of 96
10   well, poly-L-lysine coated polystyrene plates. The cells
are fixed in 0.5% glutaraldehyde in phosphate buffered
saline (PBS) for 15 minutes. Plates coated with CHO cells
are generated similarly. The cell culture supernatants are
added and antibody binding measured using a HRP-conjugated
15   goat anti-human IgG, as described above.

Two assays are used to determine if the humanized 24-
31 antibody retains its affinity to gp39 relative to the
original murine 24-31 antibody, (i) half-maximal binding
concentration and (ii) a competition assay using 50D4
20   cells. For this purpose the antibodies will be purified on
protein A and the concentration of each antibody determined
by ELISA by a comparison to isotype matched controls.
Half-maximal binding (i) are determined by incubating
humanized 24-31 with 50D4 cells at various concentrations
25   from 2 μg/ml to 0.1 ng/ml. The concentration resulting in
a half-maximal OD 490 reading, as described above, is
compared with the half-maximal binding of murine 24-31. In
the competition assay (ii) the humanized 24-31 antibody and
the murine 24-31 antibody are mixed in various molar ratios

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ranging from 100:1 to 1:100, and their ability to compete
for binding to 50D4 cells measured. Two sets are run, one
where the binding of the humanized antibody will be mea-
sured using goat-anti-human IgG (anti-mouse IgG depleted)-
5   HRP and one where the binding of murine antibody is mea-
sured using goat-anti-mouse IgG (anti-human IgG depleted)-
HRP. Binding curves, one for the murine and one for the
humanized antibody, based on molar ratios, are generated
and their relative affinities calculated. These assays
10   will confirm the anti-gp39 binding properties of the sub-
ject humanized antibodies derived from 24-31.

EXAMPLE 11

Blocking of CD40-Ig binding to gp39 by humanized 24-31.

After establishing that humanized anti-gp39 binds to
15   gp39, an assay is effected to confirm that the humanized
anti-gp39 retains its ability to block the binding of the
ligand to its receptor. For this purpose, activated human
peripheral blood T cells, or the gp39-transfected CHO
cells, 50D4, are pretreated with graded concentrations of
20   murine 24-31 or with humanized 24-31 for 15 minutes at 4°C.
Following this preincubation, CD40-Ig-biotin is added and
the binding determined by flow cytometry using PE-avidin.
Concentrations of mAbs to achieve a 50% reduction in CD40-
Ig binding are determined.

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EXAMPLE 12

Blocking of B cell proliferation and differentiation by humanized 24-31.

To confirm that humanized 24-31 blocks gp39 function,
5   B cells are cultured with a soluble fusion protein of gp39
(gp39-CD8) in the presence or absence of a range of doses
of murine 24-31 or humanized 24-31. B cell proliferative
response is assessed by ^{3}H-thymidine incorporation as shown
in **Figure 2A**.

10   T cell dependent B cell differentiation (Ig produc-
tion) is blocked by mAbs to gp39. To confirm that the
subject humanized 24-31 antibodies are effective in block-
ing the function of native gp39 expressed on the surface of
activated human T cells, the ability of the subject human-
15   ized 24-31 antibodies inhibit T cell-induced B cell differ-
entiation is assessed. B cells are co-cultured with anti-
CD3 activated T cells in the presence or absence of human-
ized 24-31 and murine 24-31. Polyclonal IgM, IgG, and IgA
production is assessed after 12 days (see **Figure 2B**).

20   These results will confirm that humanized anti-gp39 can
block CD40 binding and interfere with T-cell-dependent B
cell activation via CD40.

EXAMPLE 13

Binding Capacity

25   This experiment was effected to determine the reactiv-
ity of the murine, chimeric, and humanized (version 1) 24-
31 antibodies to the gp39 antigen relative to the concen-
tration of antibody.

Protocol:

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Plate Preparation

1. Add 50 of poly-1-lysine to each well on the 96 well plate. Incubate for 30 minutes at room temperature. Flick plates to remove poly-1-lysine.

2. Wash mgp39-CHO cells (Chinese hamster ovary cells expressing cell surface, membrane gp39) 3 times with HBSS by centrifuging at 1500 rpm for 5 minutes. Resuspend cells in HBSS to 2×10^6 cells ml.

3. Add 50 μl of cell suspension to each well and centrifuge plates at 2000 rpm for 5 minutes.

4. Add 50 μl/well of ice cold 0.5% glutaraldehyde and incubate for 15 minutes at room temperature.

5. Flick plate and blot to remove excess glutaraldehyde. Add 150 μl/well of 100 mM glycine with 0.1% BSA and incubate for 30 minutes at room temperature. Plates can be used immediately or frozen at -20°C for future use.

Binding Assay

1. Thaw plate and remove glycine buffer.

2. Serially dilute, 1:2, the test antibodies in dilution buffer starting at 1 μg/ml. Transfer 50 μg/well of each dilution in duplicate. Incubate 2 hours at room temperature.

3. Wash plate 10 times in flowing tap water.

4. Add 50 μl/well of 1:2000 dilution of goat anti-human IgG HRP or goat anti-mouse IgG HRP. Incubate 1 hour at room temperature.

5. Wash plate 10 times in flowing tap water.

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6.   Add 50 μl/well of ABTS substrate and develop plate for
     20-30 minutes. Read the plate at wavelength 405 mn
     with a background wavelength of 490 run.

7.   Plot graph of absorbance vs antibody concentration.

Results and Conclusions:

The binding capacities for the three anti-gp39 anti-
bodies (murine, chimeric and humanized version 1 of 24-31)
relative to the concentration of the antibodies, were
essentially superimposable (see **Figure 9**). This is a good
indication that these antibodies have similar binding
capacities for human gp39, indicating that the humanized
antibody has retained the gp39 binding affinity of murine
24-31.

EXAMPLE 14

**Competition Between Biotin Labeled Murine 24-31
and Chimeric and Humanized Version 1 24-31**

The ability of the chimeric and humanized (version 1)
24-31 antibodies to compete with the murine 24-31 for
binding to mgp39-CHO cells basis was evaluated. The abili-
ty of the humanized 24-31 to compete with the murine 24-31
for binding to mgp39-CHO was used to evaluate whether in
the humanized antibody the exchanges of the murine frame-
work residues with their human counterparts resulted in a
significant loss (≥ 3x decrease) of affinity.

Protocol:

Plate Preparation

1.   Add 50 of poly-1-lysine to each well on the 96 well
     plate. Incubate for 30 minutes at room temperature.
     Flick plates to remove poly-1-lysine.

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2. Wash mgp39-CHO cells 3 times with HBSS by centrifuging
    at 1500 rpm for 5 minutes. Resuspend cells in HBSS to
    2×10^6 cells/ml.

3. Add 50 μl of cell suspension to each well and centri-
    fuge plates at 2000 rpm for 5 minutes.

4. Add 50 μl/well of ice cold 0.5% glutaraldehyde and
    incubate for 15 minutes at room temperature.

5. Flick plate and blot to remove excess glutaraldehyde.
    Add 150 μl/well of 100 mM glycine with 0.1% BSA and
    incubate for 30 minutes at room temperature. Plates
    can be used immediately or frozen at -20°C for future
    use.

Competition Assay

1. Thaw plate and remove glycine buffer.

2. Dilute mouse anti-gp39 biotin to 200 ng/ml in PBS with
    1% BSA.

3. Serially dilute test antibodies (mouse, chimeric, and
    humanized 24-31) 1:2 starting at 10 μg/ml in dilution
    buffer.

4. Transfer 50 μl of diluted test antibodies and mouse
    anti-gp39 biotin into each well in duplicate. Several
    wells should contain 50 μl dilution buffer with the
    mouse anti-gp39 biotin as a maximal control group.
    Incubate 2 hours at room temperature.

5. Wash plates 10 times in flowing tap water.

6. Add 50 μl/well of 1:2000 dilution of streptavidin HRP
    and incubate 1 hour at room temperature.

7. Wash plates 10 times in flowing tap water.

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8. Add 50 μl/well of ABTS substrate and develop plate for 20-30 minutes. Read the plate at wavelength 405 nm with a background wavelength of 490 nm.

9. Percent inhibition is calculated using the average of
5   the control wells.

Results and conclusions:

All three antibodies competed equally well with the biotin labeled 24-31 (see **Figure 10**). The competition profiles are essentially superimposable at all concentra-
10 tions, within the limitations of the assay. This demon- strates that the tested humanized antibody (version 1) retains its gp39 binding affinity.

EXAMPLE 15

Modulation of T Cell Dependent B Cell Differentiation

15 To confirm that the humanized 24-31 retains the *in vitro* functional activity of murine 24-31, the humanized 24-31 was compared to the murine 24-31 in a "Lipsky" assay. Donor peripheral blood mononuclear cells were separated into two fractions, a T and a B cell fraction. The T cells
20 were first treated with mitomycin C, to prevent mitosis, and then activated with an anti-CD3 antibody. The B cells were added, together with either the murine or humanized (version 1) 24-31 antibodies. A positive control without antibody, and a negative control without B cells were
25 included in the experiment. After a 10 day incubation, the supernatants were tested for the presence of human IgM.

Protocol:

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1. Coat a 96 well plate with 50 μl/well of sterile 4 μg/ml anti-CD3 antibody (diluted in 50 mM Tris, pH 9) for 2 hours at 37°C.

2. Selectively purify T and B cells from a buffy coat using Lympho-Kwik reagents. Activate the T cells with 50 μg/ml mitomycin C per 5×10^6 cells for 30 minutes at 37°C.

3. Wash plate wells several times with sterile HBSS or media to remove non-adherent antibody.

4. Add 1×10^5 purified T cells (2×10^6/ml) to each well.

5. Add 5×10^5 purified B cells (5×10^6/ml) to each well. Add 50 μl anti-gp39 antibody (10-0.1 μg/ml) to each well in quadruplicate. Control wells should include: a) 0 antibody, b) 0 antibody, no T cells, and c) 0 antibody, no B cells.

6. Incubate plate at 37°C/5% CO_2 for 12 days.

7. Access cell growth after 7 days using 3H thymidine or any other acceptable method on duplicate wells.

8. After 12 days, collect supernatants from duplicate wells and perform ELISA assays to determine Ig production (IgM).

Results and conclusions:

The results show that the production of human IgM is inhibited 50% by the humanized 24-31 at a concentration below 0.01 μg/ml, similar to the inhibition level obtained with the murine 24-31 (see **Figure 11**). The humanized antibody retained its ability to inhibit T cell dependent B cell differentiation (IgM production) in this experiment.

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EXAMPLE 16

Evaluation of Humanized 24-31, Version 2

This experiment was conducted to determine whether humanized 24-31 version 2, as compared to version 1, has a

5   similar gp39 binding capacity in a direct binding assay.

Protocol:

Same as in Example 13 above.

Results and conclusions.

The results show that the binding capacity of the two

10   24-31 versions are essentially superimposable (see **Figure 12**). This indicates that the two versions have comparable binding activity to gp39.

EXAMPLE 17

This experiment was conducted to measure the Kd of 24-

15   31, and two humanized versions, 1 and 2.

Protocol:

A predetermined amount of each of the three antibodies (murine, version 1 or version 2 24-31) was labeled with ^{125}I using IODO-BEADS® (Pierce). Antibody bound-^{125}I was sepa-

20   rated from free ^{125}I by size separation on a Sephadex-G25/DEAE/Amberlite column.

Direct binding of the ^{125}I-labeled antibody to murine gp39-CHO cells was tested in a dilution series, in order to determine both counts/μg and the appropriate working con-

25   centration ($\approx$half-maximal binding concentration).

^{125}I-labeled antibody was mixed and incubated with non-labeled antibody in a dilution series. Based on the total amount of bound antibody and the amount of free antibody, a Scatchard plot was generated from a bound vs. bound-free

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graph. The total antibody concentration was based on a standard size of 75 kD for one active site.

The Kd was calculated by generating a "best fit" line. The inverse of the slope of the curve is the Kd. The correlation coefficient, r^2, was also computed.

Results:

The Scatchard plots were analyzed. The Kd's from this analysis are: Version 2, Kd = 14 nM; murine 24-31, Kd = 8.51 nM; version 1, Kd = 5.6. The results are depicted in Figures 13, 14 and 15, respectively. These results provide further evidence that the subject humanized antibodies bind the gp39 antigen similarly to 24-31.

Use

The humanized anti-gp39 antibodies of the present invention have potential in treating any disease condition wherein gp39 modulation and/or inhibition of the gp39-CD40 interaction is therapeutically beneficial. Moreover, the subject humanized anti-gp39 antibodies may be used in treatment of diseases wherein suppression of antibody responses to antigens are desirable. Such conditions include both autoimmune and non-autoimmune disorders.

The ability of anti-gp39 antibodies to prevent CD40 signaling in B cells is functionally translated into marked inhibition of T cell-dependent antibody responses *in vivo*. Therefore, autoimmune diseases which are mediated by auto-antibody production would be expected to benefit from anti-gp39 antibody therapy. Such diseases include systemic lupus erythematosus, idiopathic thrombocytopenic purpura, myasthenia gravis and a subpopulation of diabetic patients

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with anti-insulin and anti-insulin receptor antibodies. In
addition, CD40 signaling in B cells and dendritic cells is
essential for upregulation of co-signaling receptors such
as B7.1 and B7.2 molecules. Blocking of this CD40 signal-

5   ing by anti-gp39 antibodies interferes with antigen presen-
tation to T cells, resulting in inhibition of T cell acti-
vation and T cell-mediated responses. The therapeutic
efficacy of anti-gp39 antibodies in disease models such as
CIA, EAE, NOD mice, GVHD and graft rejection further con-

10   firms the antibody's inhibitory effect on T cell-mediated
responses. Based on this mechanism of action supported by
the efficacy in animal models, the therapeutic potential of
the subject humanized anti-gp39 antibodies extend to such
diseases as RA, MS, diabetes, psoriasis, GVHD and graft

15   rejection.

Specific conditions which are potentially treatable by
administration of the subject humanized antibodies include
the following:

Allergic bronchopulmonary aspergillosis; Autoimmune
20   hemolytic anemia; Acanthosis nigricans; Allergic contact
dermatitis; Addison's disease; Atopic dermatitis; Alopecia
areata; Alopecia universalis; Amyloidosis; Anaphylactoid
purpura; Anaphylactoid reaction; Aplastic anemia; Angioede-
ma, hereditary; Angioedema, idiopathic; Ankylosing spondy-

25   litis; Arteritis, cranial; Arteritis, giant cell; Arteri-
tis, Takayasu's; Arteritis, temporal; Asthma; Ataxia-telan-
giectasia; Autoimmune oophoritis; Autoimmune orchitis;
Autoimmune polyendocrine failure; Behcet's disease;
Berger's disease; Buerger's disease; Bullous pemphigus;

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Candidiasis, chronic mucocutaneous; Caplan's syndrome;
Post-myocardial infarction syndrome; Post-pericardiotomy
syndrome; Carditis; Celiac sprue; Chagas's disease;
Chediak-Higashi syndrome; Churg-Strauss disease; Cogan's

5   syndrome; Cold agglutinin disease; CREST syndrome; Crohn's
disease; Cryoglobulinemia; Cryptogenic fibrosing alveo-
litis; Dermatitis herpetifomis; Dermatomyositis; Diabetes
mellitus; Diamond-Blackfan syndrome; DiGeorge syndrome;
Discoid lupus erythematosus; Eosinophilic fascitis; Epi-

10   scleritis; Drythema elevatum diutinum; Erythema marginatum;
Erythema multiforme; Erythema nodosum; Familial Mediterra-
nean fever; Felty's syndrome; Fibrosis pulmonary; Glomeru-
lonephritis, anaphylactoid; Glomerulonephritis, autoimmune;
Glomerulonephritis, post-streptococcal; Glomerulonephritis,

15   post-transplantation; Glomerulopathy, membranous; Goodpas-
ture's syndrome; Graft-vs.-host disease; Granulocytopenia,
immune-mediated; Granuloma annulare; Granulomatosis, aller-
gic; Granulomatous myositis; Grave's disease; Hashimoto's
thyroiditis; Hemolytic disease of the newborn; Hemochroma-

20   tosis, idiopathic; Henoch-Schoenlein purpura; Hepatitis,
chronic active and chronic progressive; Histiocytosis X;
Hypereosinophilic syndrome; Idiopathic thrombocytopenic
purpura; Job's syndrome; Juvenile dermatomyositis; Juvenile
rheumatoid arthritis (Juvenile chronic arthritis);

25   Kawasaki's disease; Keratitis; Keratoconjunctivitis sicca;
Landry-Guillain-Barre-Strohl syndrome; Leprosy, leproma-
tous; Loeffler's syndrome; Lyell's syndrome; Lyme disease;
Lymphomatoid granulomatosis; Mastocytosis, systemic; Mixed
connective tissue disease; Mononeuritis multiplex; Muckle-

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Wells syndrome; Mucocutaneous lymph node syndrome; Mucocu-
taneous lymph node syndrome; Multicentric reticulohistiocy-
tosis; Multiple sclerosis; Myasthenia gravis; Mycosis
fungoides; Necrotizing vasculitis, systemic; Nephrotic
syndrome; Overlap syndrome; Panniculitis; Paroxysmal cold
hemoglobinuria; Paroxysmal nocturnal hemoglobinuria; Pem-
phigoid; Pemphigus; Pemphigus erythematosus; Pemphigus
foliaceus; Pemphigus vulgaris; Pigeon breeder's disease;
Pneumonitis, hypersensitivity; Polyarteritis nodosa; Poly-
myalgia rheumatica; Polymyositis; Polyneuritis, idiopathic;
Portuguese familial polyneuropathies; Pre-eclampsia/-
eclampsia; Primary biliary cirrhosis; Progressive systemic
sclerosis (Scleroderma); Psoriasis; Psoriatic arthritis;
Pulmonary alveolar proteinosis; Pulmonary fibrosis,
Raynaud's phenomenon/syndrome; Reidel's thyroiditis;
Reiter's syndrome, Relapsing polychrondritis; Rheumatic
fever; Rheumatoid arthritis; Sarcoidosis; Scleritis; Scle-
rosing cholangitis; Serum sickness; Sezary syndrome;
Sjogren's syndrome; Stevens-Johnson syndrome; Still's
disease; Subacute sclerosing panencephalitis; Sympathetic
ophthalmia; Systemic lupus erythematosus; Transplant rejec-
tion; Ulcerative colitis; Undifferentiated connective
tissue disease; Urticaria, chronic; Urticaria, cold; Uve-
itis; Vitiligo; Weber-Christian disease; Wegener's granulo-
matosis; Wiskott-Aldrich syndrome.

Of these, the preferred indications treatable or
presentable by administration of anti-gp39 antibodies
include autoimmune hemolytic anemia; aplastic anemia;
arteritis, temporal; diabetes mellitus; Felty's syndrome;

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Goodpasture's syndrome; graft-vs-host disease; idiopathic
thrombocytopenia pupura; myasthenia gravis; multiple scle-
rosis; polyarteritis nodosa; psoriasis; psoriatic arthri-
tis; rheumatoid arthritis; systemic lupus erythematosus;

5   asthma; allergic conditions; and transplant rejection.

The amount of antibody useful to produce a therapeutic
effect can be determined by standard techniques well known
to those of ordinary skill in the art. The antibodies will
generally be provided by standard technique within a phar-

10   maceutically acceptable buffer, and may be administered by
any desired route. Because of the efficacy of the present-
ly claimed antibodies and their tolerance by humans it is
possible to administer these antibodies repetitively in
order to combat various diseases or disease states within a

15   human.

The subject anti-gp39 humanized antibodies (or frag-
ments thereof) of this invention are also useful for induc-
ing immunomodulation, e.g., inducing suppression of a
human's or animal's immune system. This invention there-

20   fore relates to a method of prophylactically or therapeuti-
cally inducing immunomodulation in a human or other animal
in need thereof by administering an effective, non-toxic
amount of such an antibody of this invention to such human
or other animal.

25   The fact that the antibodies of this invention have
utility in inducing immunosuppression means that they are
useful in the treatment or prevention of resistance to or
rejection of transplanted organs or tissues (e.g., kidney,
heart, lung, bone marrow, skin, cornea, etc.); the treat-

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ment or prevention of autoimmune, inflammatory, prolifera-
tive and hyperproliferative diseases, and of cutaneous
manifestations of immunologically mediated diseases (e.g.,
rheumatoid arthritis, lupus erythematosus, systemic lupus

5   erythematosus, Hashimoto's thyroiditis, multiple sclerosis,
myasthenia gravis, type 1 diabetes, uveitis, nephrotic
syndrome, psoriasis, atopical dermatitis, contact dermati-
tis and further eczematous dermatitides, seborrheic derma-
titis, Lichen planus, Pemplugus, bullous pemphicjus, Epi-

10   dermolysis bullosa, urticaria, angioedemas, vasculitides,
erythema, cutaneous eosinophilias, Alopecia areata, etc.);
the treatment of reversible obstructive airways disease,
intestinal inflammations and allergies (e.g., Coeliac
disease, proctitis, eosinophilia gastroenteritis, mastocy-

15   tosis, Crohn's disease and ulcerative colitis) and food-
related allergies (e.g., migraine, rhinitis and eczema).
Also, the subject antibodies have potential utility for
treatment of non-autoimmune conditions wherein immunomodu-
lation is desirable, e.g., graft-versus-host disease

20   (GVHD), transplant rejection, asthma, leukemia, lymphoma,
among others.

One skilled in the art would be able, by routine
experimentation, to determine what an effective, non-toxic
amount of antibody would be for the purpose of inducing

Also, the subject antibodies can be used as immunosup-
pressants during cellular or gene therapy. This potential-
ly will enable such cells or gene therapy constructs to be

25   administered repeatedly, or at higher dosages without an
adverse immunogenic response.

One skilled in the art would be able, by routine
experimentation, to determine what an effective, non-toxic
amount of antibody would be for the purpose of inducing

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immunosuppression. Generally, however, an effective dosage
will be in the range of about 0.05 to 100 milligrams per
kilogram body weight per day.

     The antibodies of the invention may be administered to
5   a human or other animal in accordance with the aforemen-
tioned methods of treatment in an amount sufficient to
produce such effect to a therapeutic or prophylactic de-
gree. Such antibodies of the invention can be administered
to such human or other animal in a conventional dosage form
10   prepared by combining the antibody of the invention with a
conventional pharmaceutically acceptable carrier or diluent
according to known techniques. It will be recognized by
one of skill in the art that the form and character of the
pharmaceutically acceptable carrier or diluent is dictated
15   by the amount of active ingredient with which it is to be
combined, the route of administration and other well-known
variables.

     The route of administration of the antibody (or frag-
ment thereof) of the invention may be oral, parenteral, by
20   inhalation or topical. The term parenteral as used herein
includes intravenous, intramuscular, subcutaneous, rectal,
vaginal or intraperitoneal administration. The subcutane-
ous and intramuscular forms of parenteral administration
are generally preferred.

25   The daily parenteral and oral dosage regimens for
employing compounds of the invention to prophylactically or
therapeutically induce immunosuppression will generally be
in the range of about 0.05 to 100, but preferably about 0.5
to 10, milligrams per kilogram body weight per day.

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The antibody of the invention may also be administered by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or
5 a metered dose inhaler, may be prepared by conventional techniques. The preferred dosage amount of a compound of the invention to be employed is generally within the range of about 10 to 100 milligrams.

The antibody of the invention may also be administered
10 topically. By topical administration is meant non-systemic administration and includes the application of an antibody (or fragment thereof) compound of the invention externally to the epidermis, to the buccal cavity and instillation of such an antibody into the ear, eye and nose, and where it
15 does not significantly enter the blood stream. By systemic administration is meant oral, intravenous, intraperitoneal and intramuscular administration. The amount of an antibody required for therapeutic or prophylactic effect will, of course, vary with the antibody chosen, the nature and
20 severity of the condition being treated and the animal undergoing treatment, and is ultimately at the discretion of the physician. A suitable topical dose of an antibody of the invention will generally be within the range of about 1 to 100 milligrams per kilogram body weight daily.

25 Formulations

While it is possible for an antibody or fragment thereof to be administered alone, it is preferable to present it as a pharmaceutical formulation. The active ingredient may comprise, for topical administration, from

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0.001% to 10% w/w, e.g., from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w but preferably not in excess of 5% w/w and more preferably from 0.1% to 1% w/w of the formulation.

5   The topical formulations of the present invention, comprise an active ingredient together with one or more acceptable carrier(s) therefor and optionally any other therapeutic ingredients(s). The carrier(s) must be "acceptable" in the sense of being compatible with the other

10   ingredients of the formulation and not deleterious to the recipient thereof.

      Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin to the site of where treatment

15   is required, such as liniments, lotions, creams, ointments or pastes, and drops suitable for administration to the eye, ear or nose.

      Drops according to the present invention may comprise sterile aqueous or oily solutions or suspensions and may be

20   prepared by dissolving the active ingredient in a suitable aqueous solution of a bactericidal and/or fungicidal agent and/or any other suitable preservative, and preferably including a surface active agent. The resulting solution may then be clarified by filtration, transferred to a

25   suitable container which is then sealed and sterilized by autoclaving or maintaining at 90°-100°C for half an hour. Alternatively, the solution may be sterilized by filtration and transferred to the container by an aseptic technique. Examples of bactericidal and fungicidal agents suitable for

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inclusion in the drops are phenylmercuric nitrate or ace-
tate (0.002%), benzalkonium chloride (0.01%) and chlorhexi-
dine acetate (0.01%). Suitable solvents for the preparation
of an oily solution include glycerol, diluted alcohol and
5   propylene glycol.

Lotions according to the present invention include
those suitable for application to the skin or eye. An eye
lotion may comprise a sterile aqueous solution optionally
containing a bactericide and may be prepared by methods
10   similar to those for the preparation of drops. Lotions or
liniments for application to the skin may also include an
agent to hasten drying and to cool the skin, such as an
alcohol or acetone, and/or a moisturizer such as glycerol
or an oil such as castor oil or arachis oil.

15   Creams, ointments or pastes according to the present
invention are semi-solid formulations of the active ingre-
dient for external application. They may be made by mixing
the active ingredient in finely-divided or powdered form,
alone or in solution or suspension in an aqueous or non-
20   aqueous fluid, with the aid of suitable machinery, with a
greasy or non-greasy basis. The basis may comprise hydro-
carbons such as hard, soft or liquid paraffin, glycerol,
beeswax, a metallic soap; a mucilage; an oil of natural
origin such as almond, corn, arachis, castor or olive oil;
25   wool fat or its derivatives, or a fatty acid such as stea-
ric or oleic acid together with an alcohol such as propyl-
ene glycol or macrogels. The formulation may incorporate
any suitable surface active agent such as an anionic,
cationic or non-ionic surface active such as sorbitan

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esters or polyoxyethylene derivatives thereof. Suspending
agents such as natural gums, cellulose derivatives or
inorganic materials such as silicaceous silicas, and other
ingredients such as lanolin, may also be included.

5   It will be recognized by one of skill in the art that
the optimal quantity and spacing of individual dosages of
an antibody or fragment thereof of the invention will be
determined by the nature and extent of the condition being
treated, the form, route and site of administration, and

10   the particular animal being treated, and that such optimums
can be determined by conventional techniques. It will also
be appreciated by one of skill in the art that the optimal
course of treatment, i.e., the number of doses of an anti-
body or fragment thereof of the invention given per day for

15   a defined number of days, can be ascertained by those
skilled in the art using conventional course of treatment
determination tests.

        Without further elaboration, it is believed that one
skilled in the art can, using the preceding description,

20   utilize the present invention to its fullest extent. The
following are, therefore, to be construed as merely illus-
trative examples and not a limitation of the scope of the
present invention in any way.

Capsule Composition

25   A pharmaceutical composition of this invention in the
form of a capsule is prepared by filling a standard two-
piece hard gelatin capsule with 50 mg. of an antibody or
fragment thereof of the invention, in powdered form, 100

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mg. of lactose, 32 mg. of talc and 8 mg. of magnesium stearate.

Injectable Parenteral Composition

A pharmaceutical composition of this invention in a form suitable for administration by injection is prepared by stirring 1.5k by weight of an antibody or fragment thereof of the invention in 10k by volume propylene glycol and water. The solution is sterilized by filtration.

Ointment Composition

Antibody or fragment thereof of the invention 1.0 g.

White soft paraffin to 100.0 g.

The antibody or fragment thereof of the invention is dispersed in a small volume of the vehicle to produce a smooth, homogeneous product. Collapsible metal tubes are then filled with the dispersion.

Topical Cream Composition

Antibody or fragment thereof of the invention 1.0 g.

Polawax GP 200 20.0 g.

Lanolin Anhydrous 2.0 g.

White Beeswax 2.5 g.

Methyl hydroxybenzoate 0.1 g.

Distilled Water to 100.0 g.

The polawax, beeswax and lanolin are heated together at 60°C. A solution of methyl hydroxybenzoate is added and homogenization is achieved using high speed stirring. The temperature is then allowed to fall to SOOC. The antibody or fragment thereof of the invention is then added and dispersed throughout, and the composition is allowed to cool with slow speed stirring.

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Topical Lotion Composition

      Antibody or fragment thereof of the invention 1.0 g.

      Sorbitan Monolaurate 0.6 g. Polysorbate 20 0.6 g.

      Cetostearyl Alcohol 1.2 g. Glycerin 6.0 g.

5   Methyl Hydroxybenzoate 0.2 g.

      Purified Water B.P. to 100.00 ml. (B.P. = British

Pharmacopeia)

      The methyl hydroxybenzoate and glycerin are dissolved

in 70 ml. of the water at 75°C. The sorbitan monolaurate,

10   polysorbate 20 and cetostearyl alcohol are melted together

at 75°C and added to the aqueous solution. The resulting

emulsion is homogenized, allowed to cool with continuous

stirring and the antibody or fragment thereof of the inven-

tion is added as a suspension in the remaining water. The

15   whole suspension is stirred until homogenized.

Eye Drop Composition

      Antibody or fragment thereof of the invention 0.5 g.

      Methyl Hydroxybenzoate 0.01 g.

      Propyl Hydroxybenzoate 0.04 g.

20   Purified Water B.P. to 100.00 ml.

      The methyl and propyl hydroxybenzoates are dissolved

in 70 ml. purified water at 75°C and the resulting solution

is allowed to cool. The antibody or fragment thereof of

the invention is then added, and the solution is sterilized

25   by filtration through a membrane filter (0.022 Am pore

size), and packed aseptically into suitable sterile con-

tainers.

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<u>Composition for Administration by Inhalation</u>

     For an aerosol container with a capacity of 15-20 ml:
mix 10 mg. of an antibody or fragment thereof of the inven-
tion with 0.2-0.5k of a lubricating agent, such as polysor-
5   bate 85 or oleic acid, and disperse such mixture in a
propellant, such as freon, preferably in a combination of
(1,2 dichlorotetrafluoroethane) and difluorochloromethane
and put into an appropriate aerosol container adapted for
either intranasal or oral inhalation administration.

10   Composition for Administration by Inhalation For an aerosol
container with a capacity of 15-20 ml: dissolve 10 mg. of
an antibody or fragment thereof of the invention in ethanol
(6-8 ml.), add 0.1-0.2k of a lubricating agent, such as
polysorbate 85 or oleic acid; and disperse such in a pro-
15   pellant, such as freon, preferably in combination of (1-2
dichlorotetrafluoroethane) and difluorochloromethane, and
put into an appropriate aerosol container adapted for
either intranasal or oral inhalation administration.

     The antibodies and pharmaceutical compositions of the
20   invention are particularly useful for parenteral adminis-
tration, i.e., subcutaneously, intramuscularly or intrave-
nously. The compositions for parenteral administration
will commonly comprise a solution of an antibody or frag-
ment thereof of the invention or a cocktail thereof dis-
25   solved in an acceptable carrier, preferably an aqueous
carrier. A variety of aqueous carriers may be employed,
e.g., water, buffered water, 0.4k saline, 0.3% glycine, and
the like. These solutions are sterile and generally free
of particulate matter. These solutions may be sterilized

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by conventional, well-known sterilization techniques. The
compositions may contain pharmaceutically acceptable auxil-
iary substances as required to approximate physiological
conditions such as pH adjusting and buffering agents, etc.
5   The concentration of the antibody or fragment thereof of
the invention in such pharmaceutical formulation can vary
widely, i.e., from less than about 0.5k, usually at or at
least about 1% to as much as 15 or 20% by weight, and will
be selected primarily based on fluid volumes, viscosities,
10   etc., according to the particular mode of administration
selected.

Thus, a pharmaceutical composition of the invention
for intramuscular injection could be prepared to contain 1
mL sterile buffered water, and 50 mg. of an antibody or
15   fragment thereof of the invention. Similarly, a pharmaceu-
tical composition of the invention for intravenous infusion
could be made up to contain 250 ml. of sterile Ringer's
solution, and 150 mg. of an antibody or fragment thereof of
the invention. Actual methods for preparing parenterally
20   administrable compositions are well-known or will be appar-
ent to those skilled in the art, and are described in more
detail in, e.g., *Remington's Pharmaceutical Science*, 15th
ed., Mack Publishing Company, Easton, PA, hereby incorpo-
rated by reference herein.

25   The antibodies (or fragments thereof) of the invention
can be lyophilized for storage and reconstituted in a
suitable carrier prior to use. This technique has been
shown to be effective with conventional immune globulins

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and art-known lyophilization and reconstitution techniques can be employed.

Depending on the intended result, the pharmaceutical composition of the invention can be administered for pro-

5   phylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. In prophylactic applications, compositions

10 containing the present antibodies or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance.

Single or multiple administrations of the pharmaceutical compositions can be carried out with dose levels and

15 pattern being selected by the treating physician. In any event, the pharmaceutical composition of the invention should provide a quantity of the altered antibodies (or fragments thereof) of the invention sufficient to effectively treat the patient.

20 It should also be noted that the antibodies of this invention may be used for the design and synthesis of either peptide or non-peptide compounds (mimetics) which would be useful in the same therapy as the antibody. *See*, e.g., Saragovi et al, *Science*, 253:792-795 (1991).

25 From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without diverting from the scope

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of the invention.   Accordingly, the invention is not limit-
ed by the appended claims.

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WHAT IS CLAIMED IS:

1.　A humanized antibody which is capable of competing with the murine 24-31 antibody for inhibiting CD40 binding to gp39.

5　　2.　The antibody of claim 1 which contains the complementarity determining regions of the 24-31 antibody as set forth in Figures 4-8 or variants and equivalents which contain one or more conservative amino acid substitutions.

3.　A humanized antibody derived from murine monoclonal antibody 24-31.

4.　A humanized antibody derived from murine monoclonal antibody 24-31 which retains at least about one-third the gp39 antigen binding affinity of the murine 24-31 antibody.

5.　A humanized antibody derived from murine monoclonal antibody 24-31 which retains the half-maximal potency in *in vitro* functional activity in a B cell assay at a concentration of not more than three times the concentration of the parent murine 24-31 antibody.

6.　The method of Claim 5, wherein the B-cell assay measures T-cell dependent antibody production.

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7.   The humanized antibody of Claim 1, wherein said antibody contains a humanized variable light sequence selected from the following group:

(1)   DIVMTQSPSFLSASVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
5   GVPDRFSGSGSGTDFTLTISSLQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(2)   DIVMTQSPDSLAVSLGERATINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
           GVPDRFSGSGSGTDFTLTISSLQAEDVADYFC QQYNSYPYT FGGGTKLEIK;

(3)   DIVMTQSPSFMSTSVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
           GVPDRFSGSGSGTDFTLTISSMQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

10   (4)   DIVMTQSPDSMATSLGERVTINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
           GVPDRFSGSGSGTDFTLTISSMQAEDVADYFC QQYNSYPYT FGGGTKLEIK,

and variants and equivalents thereof which contain one or more conservative amino acid substitutions which do not substantially affect the ability of the resultant humanized
15   antibody to bind the gp39 antigen.

8.   The humanized antibody of Claim 1, wherein said antibody contains a humanized variable heavy sequence selected from the group consisting of:

(1)   EVQLQESGPGLVKPSETLSLTCTVSGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
20   RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(2)   EVQLQESGPGLVKPSQTLSLTCTVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
           RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(3)   EVQLQESGPGLVKPSQTLSLTCAVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSNNQFSLNLNSVTRADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(4)   EVQLQESGPGLVKPSETLSLTCAVYGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFYLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS.

and variants and equivalents thereof which contain one or more
conservative amino acid substitutions which do not substantial-
ly affect the ability of the resultant humanized antibody to
bind the gp39 antigen.

10   9.   The humanized antibody of Claim 1, which contains a
humanized variable light sequence selected from the group
consisting of the following group:

(1)   DIVMTQSPSFLSASVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSLQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

15   (2)   DIVMTQSPDSLAVSLGERATINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSLQAEDVADYFC QQYNSYPYT FGGGTKLEIK;

(3)   DIVMTQSPSFMSTSVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSMQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(4)   DIVMTQSPDSMATSLGERVTINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
20   GVPDRFSGSGSGTDFTLTISSMQAEDVADYFC QQYNSYPYT FGGGTKLEIK

and a humanized variable heavy sequence selected from the
following group:

(1)   EVQLQESGPGLVKPSETLSLTCTVSGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

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    (2)   EVQLQESGPGLVKPSQTLSLTCTVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

    (3)   EVQLQESGPGLVKPSQTLSLTCAVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSNNQFSLNLNSVTRADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

5

    (4)   EVQLQESGPGLVKPSETLSLTCAVYGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFYLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS, and

and variants and equivalents thereof which contain one or more
conservative amino acid substitutions which do not substantial-
10   ly affect the ability of the resultant humanized antibody to
bind the gp39 antigen.

    10.   The humanized antibody of Claim 5, which contains
humanized variable light sequence (1) and humanized variable
heavy sequence (1).

15   11.   The humanized antibody of Claim 5, which contains
humanized variable light sequence (2) and humanized variable
heavy sequence (1).

    12.   The humanized antibody of Claim 5, which contains
humanized variable light sequence (1) and humanized variable
20   heavy sequence (2).

    13.   The humanized antibody of Claim 5, which contains
humanized variable light sequence (2) and humanized variable
heavy sequence (2).

14. The humanized antibody of Claim 1, which contains the human kappa or lambda light chain constant region and either the human gamma 1 or gamma 4 heavy chain constant region.

15. A DNA sequence which encodes for a humanized antibody according to Claim 1.

16. An expression vector which contains a DNA sequence according to Claim 10.

17. A pharmaceutical composition which contains a humanized antibody derived from murine monoclonal antibody 24-31.

18. The pharmaceutical composition of Claim 17, wherein said humanized antibody contains a humanized variable light sequence selected from the following group:

  (1) DIVMTQSPSFLSASVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSLQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

  (2) DIVMTQSPDSLAVSLGERATINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSLQAEDVADYFC QQYNSYPYT FGGGTKLEIK;

  (3) DIVMTQSPSFMSTSVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSMQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

  (4) DIVMTQSPDSMATSLGERVTINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
       GVPDRFSGSGSGTDFTLTISSMQAEDVADYFC QQYNSYPYT FGGGTKLEIK.

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and variants and equivalents thereof which contain one or more
conservative amino acid substitutions which do not substantial-
ly affect the ability of the resultant humanized antibody to
bind the gp39 antigen.

5   19.   The pharmaceutical composition of Claim 18, wherein
said humanized antibody contains a humanized variable heavy
sequence selected from the following group:

(1)   EVQLQESGPGLVKPSETLSLTCTVSGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

10   (2)   EVQLQESGPGLVKPSQTLSLTCTVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(3)   EVQLQESGPGLVKPSQTLSLTCAVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSNNQFSLNLNSVTRADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

15   (4)   EVQLQESGPGLVKPSETLSLTCAVYGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
       RISISRDTSKNQFYLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS,

and variants and equivalents thereof which contain one or more
conservative amino acid substitutions which do not substantial-
ly affect the ability of the resultant humanized antibody to
20   bind the gp39 antigen.

20   The pharmaceutical composition of Claim 17, wherein
said humanized antibody contains a humanized variable light
sequence selected from the following group:

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(1)   DIVMTQSPSFLSASVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSLQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(2)   DIVMTQSPDSLAVSLGERATINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSLQAEDVADYFC QQYNSYPYT FGGGTKLEIK;

5   (3)   DIVMTQSPSFMSTSVGDRVTITC KASQNVITAVA WYQQKPGKSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSMQPEDFADYFC QQYNSYPYT FGGGTKLEIK;

(4)   DIVMTQSPDSMATSLGERVTINC KASQNVITAVA WYQQKPGQSPKLLIY SASNRYT
GVPDRFSGSGSGTDFTLTISSMQAEDVADYFC QQYNSYPYT FGGGTKLEIK

and a humanized variable heavy sequence selected from the

10   following group:

(1)   EVQLQESGPGLVKPSETLSLTCTVSGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(2)   EVQLQESGPGLVKPSQTLSLTCTVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFSLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

15   (3)   EVQLQESGPGLVKPSQTLSLTCAVSGDSIT NGFWI WIRKHPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSNNQFSLNLNSVTRADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS;

(4)   EVQLQESGPGLVKPSETLSLTCAVYGDSIT NGFWI WIRKPPGNKLEYMG YISYSGSTYYNPSLKS
RISISRDTSKNQFYLKLSSVTAADTGVYYCAC RSYGRTPYYFDF WGQGTTLTVSS,

20   and variants and equivalents thereof which contain one or more
conservative amino acid substitutions which do not substantial-
ly affect the ability of the resultant humanized antibody to
bind the gp39 antigen.

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21. The pharmaceutical composition of Claim 20, wherein the humanized antibody contains humanized variable light sequence (1) and variable heavy sequence (1).

22. The pharmaceutical composition of Claim 20, wherein the antibody contains humanized variable light sequence (2) and humanized variable heavy sequence (1).

23. The pharmaceutical composition of Claim 20, which contains humanized variable light sequence (1) and humanized variable heavy sequence (2).

24. The pharmaceutical composition of Claim 20, which contains humanized variable light sequence (2) and humanized variable heavy sequence (2).

25. A method of treatment of a disease treatable by modulating gp39 expression or inhibiting the gp39/CD40 interaction which comprises administering a therapeutically effective amount of a humanized antibody according to Claim 1.

26. The method of Claim 25, wherein said disease is an autoimmune disorder.

27. The method of Claim 21, wherein said autoimmune disorder is selected from the group consisting of rheumatoid arthritis, psoriasis multiple sclerosis, diabetes, systemic lupus erythematosus and ITP.

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28.   The method of Claim 25, wherein the disease is a non-
autoimmune disorder.

29.   The method of Claim 27, wherein the disease is graft-
versus-host disease or graft rejection.

5   30.   A method of suppressing humoral and/or cellular
immune responses against cells or vectors administered during
cell or gene therapy comprising further administering prior,
during or after gene therapy an amount of a humanized antibody
derived from murine antibody 24-31 sufficient to suppress

10   humoral and/or cellular immune responses against the cell or
vector used during cell or gene therapy.

31.   The method of Claim 30, wherein the vector is a viral
vector, a DNA or an antisense RNA.

32.   The method of Claim 31, wherein the viral vector is

15   an adenovirus or retrovirus.

33.   The method of Claim 30, wherein said humanized
antibody contains a sequence set forth in at least one Figures
5-8.

34.   An improved method of treatment which involves the

20   transplantation of cells, tissues or organs of the same or
different species into a subject in need of such treatment,
wherein the improvement comprises administering a humanized
anti-human gp39 antibody derived from murine antibody 24-31;

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prior, during or after transplantation; in an amount sufficient
to suppress immune responses against said transplanted cell,
tissue or organ or to suppress immune responses elicited by the
transplanted cell, tissue or organ against the host.

FIG. 1

Anti-GP39 HU24-31 in N5KG1 (Version #1)
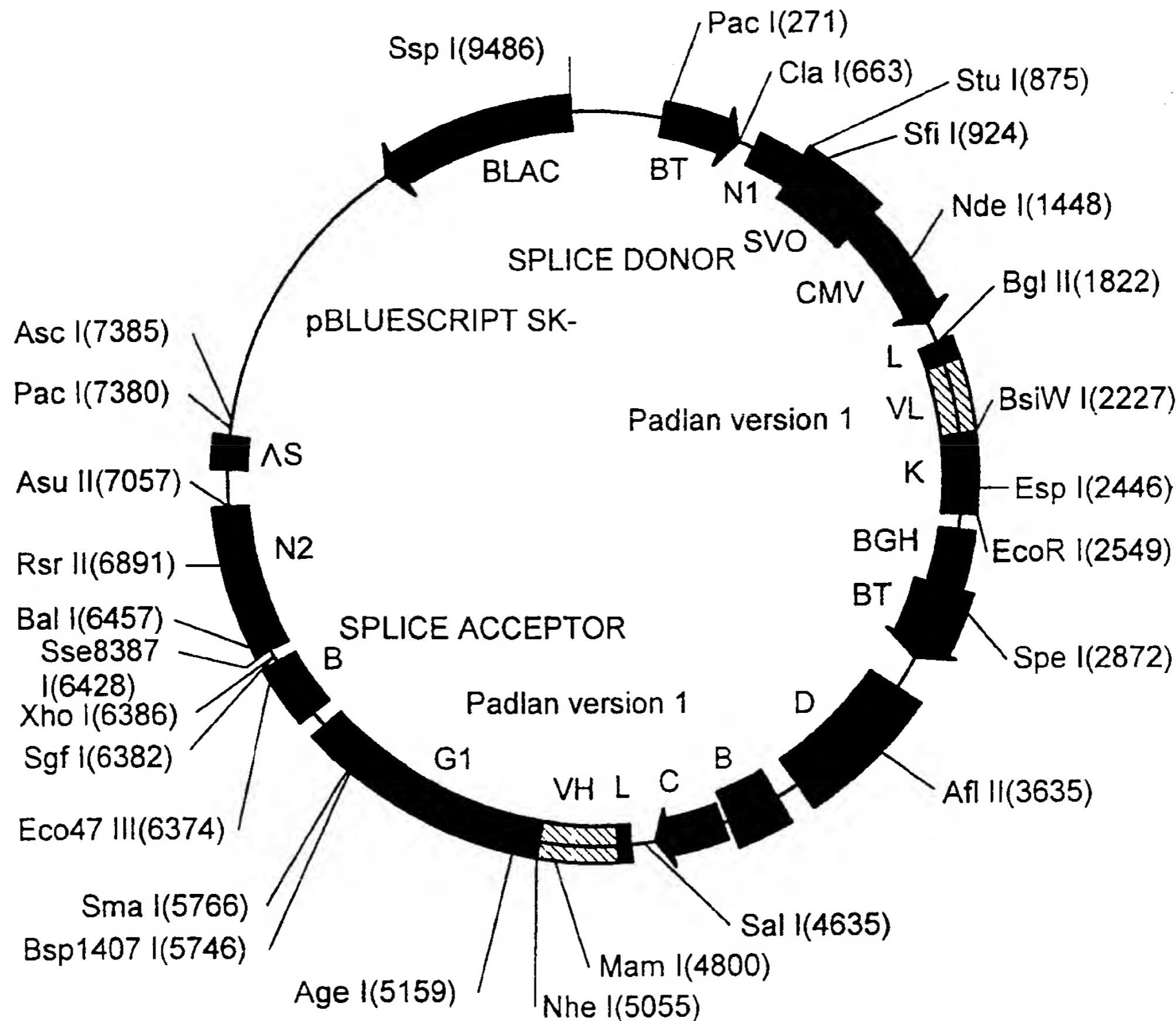
9597 BP   Map BY Mitchell Raff   06/01/95

C = Cytomegalovirus promoter/enhancer   L = Leader

BT = Mouse Beta globin major promoter   SVO = SV40 origin

B = Bovine growth hormone polyadenylation   SV = SV40 polyadenylation

N1 = Neomycin phosphotransferase exon 1   D = Dihydrofolate Reductase

N2 = Neomycin phosphotransferase exon 2   BLAC = Betalactamase gene

K = Human immunonoglobulin kappa constant region

G1 = Human immunonoglobulin gamma 1 constant region

VL = Anti-GP39 variable light region (version #1)

VH = Anti-GP39 variable heavy region (version #1)

NONCUTTERS = AvrII, Bst1107I, DraIII, FseI, HindIII, I-PpoI, I-SceI,

KpnI, MluI, MunI, PmeI, PmlI, SgrAI, SrfI, Swa I, XbaI, XcmI

N5KG1 cut BglII + BsiWI and VL dropped in & cut SalI + NheI and VH dropped in.

FIG. 2
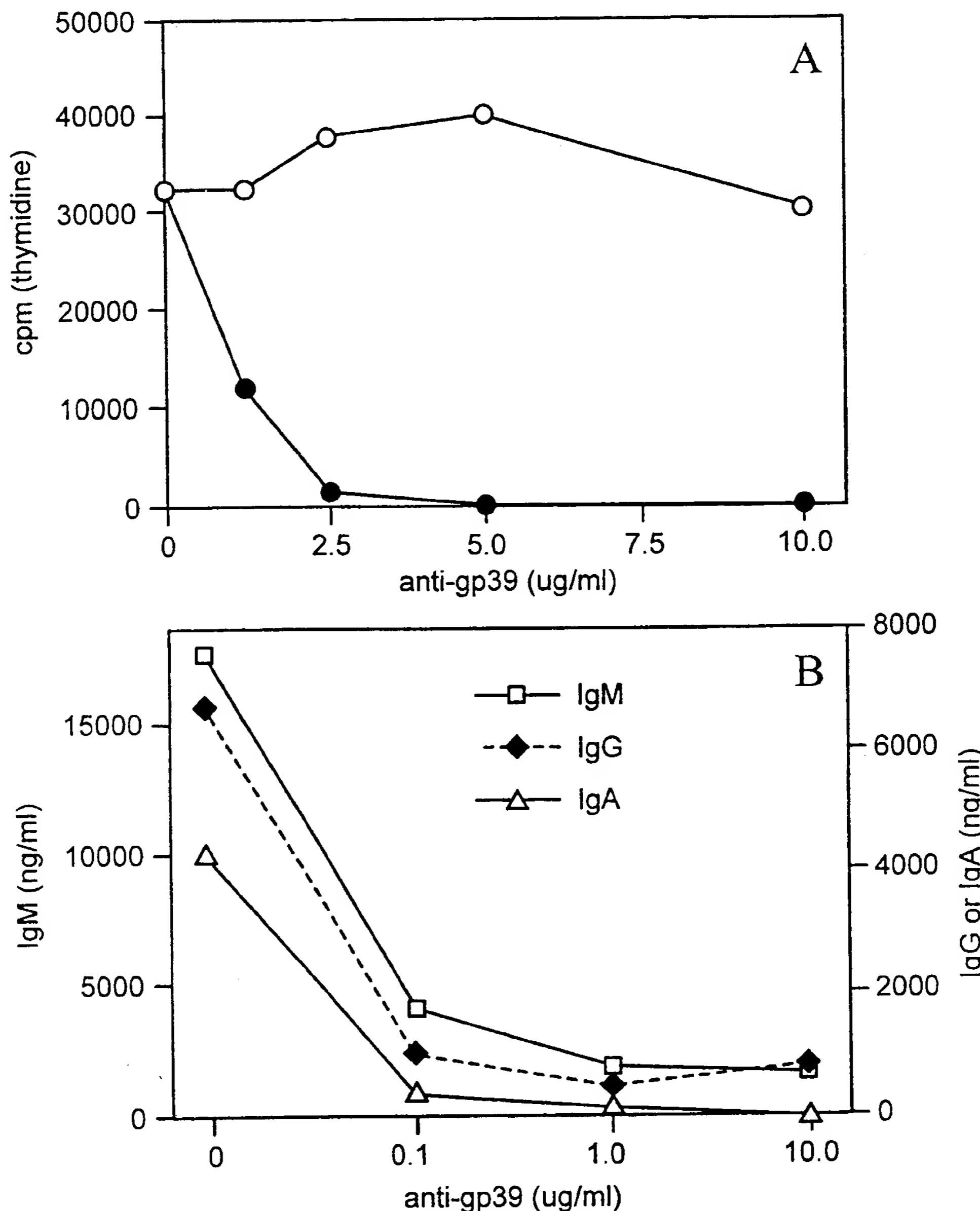
Anti-gp39 inhibits B cell proliferation and differentiation, but not allogeneic T cell proliferation.   A. Human PBL were cultured in 96 well plates (0.1 x 10^6/well) in the presence or absence of the 20% (v/v) soluble gp39-CD8 (sgp39-CD8) fusion protein and 5 ug/ml rhuIL-4 for 3 d. Anti-gp39 mAb, 24-31 (●), or a control murine IgG1 mAb (○), were added at a range of concentrations (1.25-10 ug/ml). Cultures with pulsed with 1 uCi ^{3}H-thymidine during the final 6 hr of a 72 hr culture period.   B. Mitomycin treated T cells (5 x 10^4/well) activated with immobilized anti-CD3 (64.1) were cultured with 2.5 x 10^4/well IgD$^+$ B cells in 96-well microtiter plates for 12d in the presence or absence of various concentrations (0.1-10.0 ug/ml) of anti-gp39 mAb, 24-31. Culture supernatants were subsequently assayed for IgM (□), IgG (◆), and IgA (△) by isotype specific ELISA.

FIG. 3

Fluorescence/FL1
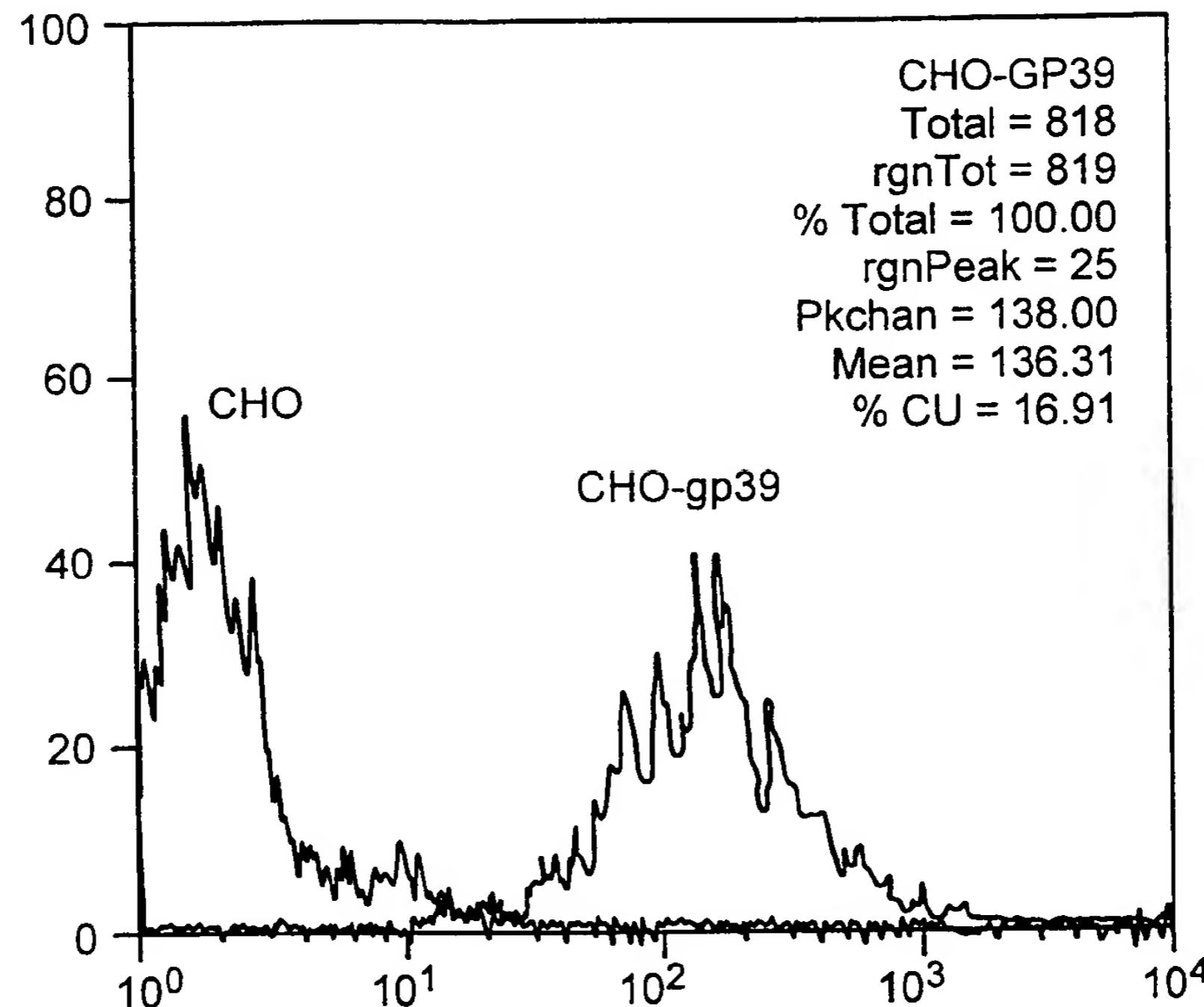
FACS analysis of non-transfected CHO cells and a gp39 transfectant.
1×10^6 cells were treated with the mouse anti-gp39 antibody 24-31 and
then with a goat-anti-mouse IgG-FITC conjugate (Southern Biotechnology
Associates). The samples were analyzed on FACScan (Becton Dickenson).

24-31 Humanized V_L #1

```
      BglII        9                  18                  27                  36                  45                  54
5'  AGA TCT  CTC ACC | ATG GGC TTC AAG ATG GAG TCA CAG TTT CTG GCC TTT GTA TTC
    --- --- --- --- | --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    |  M   G   F   K   M   E   S   Q   F   L   A   F   V   F

                    63                  72                  81      FR1 90                  99                 108
    GCG TTT CTC TGG TTG TCT GGT GTT GAT GGA | GAC ATT GTG ATG ACC CAG TCT CCA
    --- --- --- --- --- --- --- --- --- --- | --- --- --- --- --- --- --- ---
     A   F   L   W   L   S   G   V   D   G  |  D   I   V   M   T   Q   S   P

                   117                 126                 135                 144                 153 CDR1          162
    TCT TTC CTC TCC GCC TCC GTA GGA GAC AGG GTC ACC ATC ACC TGC | AAG GCC AGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- | --- --- ---
     S   F   L   S   A   S   V   G   D   R   V   T   I   T   C  |  K   A   S

                   171                 180             | 189 FR2     198                 207                 216
    CAG AAT GTG ATT ACT GCT GTA GCC | TGG TAT CAA CAG AAA CCA GGA AAG TCT CCT
    --- --- --- --- --- --- --- --- | --- --- --- --- --- --- --- --- --- ---
     Q   N   V   I   T   A   V   A  |  W   Y   Q   Q   K   P   G   K   S   P

                   225             | 234 CDR2          243                 252 | FR3 261                 270
    AAA TTG CTG ATT TAC | TCG GCA TCC AAT CGG TAC ACT | GGA GTC CCT GAT CGC TTC
    --- --- --- --- --- | --- --- --- --- --- --- --- | --- --- --- --- --- ---
     K   L   L   I   Y  |  S   A   S   N   R   Y   T  |  G   V   P   D   R   F

                   279                 288                 297                 306                 315                 324
    TCA GGC AGT GGG TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC TCT CTC CAG CCA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     S   G   S   G   S   G   T   D   F   T   L   T   I   S   S   L   Q   P

                   333                 342             | 351 CDR3    360                 369                 378
    GAA GAC TTC GCA GAT TAT TTC TGC | CAG CAA TAT AAC AGC TAT CCG TAC ACG | TTC
    --- --- --- --- --- --- --- --- | --- --- --- --- --- --- --- --- --- | ---
     E   D   F   A   D   Y   F   C  |  Q   Q   Y   N   S   Y   P   Y   T  |  F

     FR4 387                 396                 405   BsiWI
    GGA GGG GGG ACC AAG CTG GAA ATC AAA CGT ACG 3'
    --- --- --- --- --- --- --- --- --- --- ---
     G   G   G   T   K   L   E   I   K   R   T
```

FIGURE 4

24-31 Humanized V$_L$ #2

```
     BglII    9                  18              27              36              45              54
5'  AGA TCT CTC ACC│ATG GGC TTC AAG ATG GAG TCA CAG TTT CTG GCC TTT GTA TTC
    --- --- --- ---│--- --- --- --- --- --- --- --- --- --- --- --- --- ---
                    │ M   G   F   K   M   E   S   Q   F   L   A   F   V   F

           63              72              81              90  FR1         99             108
    GCG TTT CTC TGG TTG TCT GGT GTT GAT GGA│GAC ATT GTG ATG ACC CAG TCT CCA
    --- --- --- --- --- --- --- --- --- ---│--- --- --- --- --- --- --- ---
     A   F   L   W   L   S   G   V   D   G │ D   I   V   M   T   Q   S   P

           117             126             135             144         153  CDR1        162
    GAT TCT CTC GCC GTG TCC CTC GGA GAG AGG GCC ACC ATC AAC TGC│AAG GCC AGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- ---│--- --- ---
     D   S   L   A   V   S   L   G   E   R   A   T   I   N   C │ K   A   S

           171             180         │189  FR2     198          ·    207             216
    CAG AAT GTG ATT ACT GCT GTA GCC│TGG TAT CAA CAG AAA CCA GGA CAA TCT CCT
    --- --- --- --- --- --- --- ---│--- --- --- --- --- --- --- --- --- ---
     Q   N   V   I   T   A   V   A │ W   Y   Q   Q   K   P   G   Q   S   P

           225         │234  CDR2    243             252 │    FR3 261             270
    AAA TTG CTG ATT TAC│TCG GCA TCC AAT CGG TAC ACT│GGA GTC CCT GAT CGC TTC
    --- --- --- --- ---│--- --- --- --- --- ---│--- --- --- --- --- ---
     K   L   L   I   Y │ S   A   S   N   R   Y   T │ G   V   P   D   R   F

           279             288             297             306             315       '   324
    TCA GGC AGT GGG TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC TCT CTC CAG GCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     S   G   S   G   S   G   T   D   F   T   L   T   I   S   S   L   Q   A

           333             342         │351  CDR3    360             369         │378
    GAA GAC GTG GCA GAT TAT TTC│TGC CAG CAA TAT AAC AGC TAT CCG TAC ACG│TTC
    --- --- --- --- --- --- ---│--- --- --- --- --- --- --- --- ---│---
     E   D   V   A   D   Y   F │ C   Q   Q   Y   N   S   Y   P   Y   T │ F

       FR4 387             396             405  BsiWI
    GGA GGG GGG ACC AAG CTG GAA ATC AAA CGT ACG 3'
    --- --- --- --- --- --- --- --- --- --- ---
     G   G   G   T   K   L   E   I   K   R   T
```

FIGURE 5

24-31 Humanized V_H #1

```
        SalI        9              18              27              36              45              54
5'  GTC GAC ATG ATG GTG TTA AGT CTT CTG TAC CTG TTG ACA GCC CTT CCG GGT TTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
            M   M   V   L   S   L   L   Y   L   L   T   A   L   P   G   F

            63  FR1         72              81              90              99              108
    CTG TCA GAG GTG CAG CTT CAG GAG TCA GGA CCT GGC CTC GTG AAA CCT TCT GAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     L   S   E   V   Q   L   Q   E   S   G   P   G   L   V   K   P   S   E

            117             126             135             144             153 CDR1        162
    ACT CTG TCC CTC ACC TGT ACC GTC TCT GGC GAC TCC ATC ACT AAT GGT TTC TGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     T   L   S   L   T   C   T   V   S   G   D   S   I   T   N   G   F   W

            171 FR2         180             189             198             207 CDR2    216
    ATC TGG ATC CGG AAA CCA CCA GGG AAT AAA CTT GAG TAC ATG GGC TAC ATA AGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     I   W   I   R   K   P   P   G   N   K   L   E   Y   M   G   Y   I   S

            225             234             243             252             261 FR3        270
    TAC AGT GGT AGC ACT TAC TAC AAT CCA TCT CTC AAG AGT CGA ATC TCC ATC TCT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     Y   S   G   S   T   Y   Y   N   P   S   L   K   S   R   I   S   I   S

            279             288             297             306             315             324
    CGC GAC ACA TCC AAG AAC CAG TTC TCT CTA AAG TTG TCT TCT GTG ACT GCC GCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     R   D   T   S   K   N   Q   F   S   L   K   L   S   S   V   T   A   A

            333             342             351 CDR3 360             369             378
    GAC ACA GGC GTG TAT TAC TGT GCC TGC CGC AGT TAC GGG AGG ACC CCG TAC TAC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     D   T   G   V   Y   Y   C   A   C   R   S   Y   G   R   T   P   Y   Y

            387 FR4         396             405             414   NheI
    TTT GAC TTC TGG GGC CAA GGC ACC ACT CTC ACC GTC TCC TCA GCT AGC 3'
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     F   D   F   W   G   Q   G   T   T   L   T   V   S   S   A   S
```

FIGURE 6

Anti-gp39 24-31 V$_K$ Sequence

```
      Bgl II          9                18              27          36              45              54
5'   AGA  TCT  CTC  ACC  ATG  GGC  TTC  AAG  ATG  GAG  TCA  CAG  TTT  CTG  GCC  TTT  GTA  TTC
     ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
                         M    G    F    K    M    E    S    Q    F    L    A    F    V    F

               63                72              81   |+1   90   FRI        99              108
     GCG  TTT  CTC  TGG  TTG  TCT  GGT  GTT  GAT  GGA | GAC  ATT  GTG  ATG  ACC  CAG  TCT  CAA
     ---  ---  ---  ---  ---  ---  ---  ---  ---  --- | ---  ---  ---  ---  ---  ---  ---  ---
      A    F    L    W    L    S    G    V    D    G  | D    I    V    M    T    Q    S    Q

               117               126             135            144            153|   CDR1  162
     AAA  TTC  ATG  TCC  ACA  TCC  GTA  GGA  GAC  AGG  GTC  AGC  ATC  ACC  TGC|  AAG  GCC  AGT
     ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
      K    F    M    S    T    S    V    G    D    R    V    S    I    T    C|   K    A    S

               171               180            |189   FR2   198            207             216
     CAG  AAT  GTG  ATT  ACT  GCT  GTA  GCC  |  TGG  TAT  CAA  CAG  AAA  CCA  GGA  CAA  TCT  CCT
     ---  ---  ---  ---  ---  ---  ---  --- |   ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
      Q    N    V    I    T    A    V    A |    W    Y    Q    Q    K    P    G    Q    S    P

               225              |234   CDR2  243            252|   FR3  261             270
     AAA  TTG  CTG  ATT  TAC|   TCG  GCA  TCC  AAT  CGG  TAC  ACT|  GGA  GTC  CCT  GAT  CGC  TTC
     ---  ---  ---  ---  --- |   ---  ---  ---  ---  ---  ---  --- |  ---  ---  ---  ---  ---  ---
      K    L    L    I    Y|    S    A    S    N    R    Y    T|   G    V    P    D    R    F

               279              288             297            306             315             324
     TCA  GGC  AGT  GGG  TCT  GGG  ACA  GAT  TTC  ACT  CTC  ACC  ATC  AGC  AAT  ATG  CAG  TCT
     ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
      S    G    S    G    S    G    T    D    F    T    L    T    I    S    N    M    Q    S

               333              342            |351  CDR3  360            369            |378
     GAA  GAC  CTG  GCA  GAT  TAT  TTC  TGC|   CAG  CAA  TAT  AAC  AGC  TAT  CCG  TAC  ACG|  TTC
     ---  ---  ---  ---  ---  ---  ---  --- |   ---  ---  ---  ---  ---  ---  ---  ---  --- |  ---
      E    D    L    A    D    Y    F    C|    Q    Q    Y    N    S    Y    P    Y    T|   F

     FR4  387              396            405|  Bsi WI
     GGA  GGG  GGG  ACC  AAG  CTG  GAA  ATC  AAA  CGT  ACG  3'
     ---  ---  ---  ---  ---  ---  ---  ---  ---  ---  ---
      G    G    G    T    K    L    E    I    K    R    T
```

FIGURE 7

Anti gp39 24-31 V$_H$ Sequence

```
     SalI            9              18             27             36             45             54
5'   GTC GAC ATG ATG GTG TTA AGT CTT CTG TAC CTG TTG ACA GCC CTT CCG GGT TTC
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
              M   M   V   L   S   L   L   Y   L   L   T   A   L   P   G   F

                 +1
                 63          FRI 72            81             90             99            108
     CTG TCA GAG GTG CAG CTT CAG GAG TCA GGA CCT AGC CTC GTG AAA CCT TCT CAG
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
      L   S   E   V   Q   L   Q   E   S   G   P   S   L   V   K   P   S   Q

             117            126            135            144            153 CDR1       162
     ACT CTG TCC CTC ACC TGT TCT GTC ACT GGC GAC TCC ATC ACT AAT GGT TTC TGG
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
      T   L   S   L   T   C   S   V   T   G   D   S   I   T   N   G   F   W

             171 FR2        180            189            198      .      207     CDR2 216
     ATC TGG ATC CGG AAA TTC CCA GGG AAT AAA CTT GAG TAC ATG GGC TAC ATA AGT
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
      I   W   I   R   K   F   P   G   N   K   L   E   Y   M   G   Y   I   S
                                                                                    FR3
             225            234            243            252            261        270
     TAC AGT GGT AGC ACT TAC TAC AAT CCA TCT CTC AAG AGT CGA ATC TCC ATC ACT
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
      Y   S   G   S   T   Y   Y   N   P   S   L   K   S   R   I   S   I   T

             279            288            297            306            315        324
     CGC GAC ACA TCC CAG AAC CAG TTC TAC CTA CAA TTG AAT TCT GTG ACT ACT GAG
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
      R   D   T   S   Q   N   Q   F   Y   L   Q   L   N   S   V   T   T   E

             333            342            351 CDR3       360            369        378
     GAC ACA GGC ACA TAT TAC TGT GCC TGC CGC AGT TAC GGG AGG ACC CCG TAC TAC
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
      D   T   G   T   Y   Y   C   A   C   R   S   Y   G   R   T   P   Y   Y

             387 FR4        396            405            414            423 NheI
     TTT GAC TTC TGG GGC CAA GGC ACC ACT CTC ACC GTC TCC TCA GCT AGC 3'
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
      F   D   F   W   G   Q   G   T   T   L   T   V   S   S   A   S
```

FIGURE 8

FIG. 9

Direct Binding of Anti-gp39 Antibodies to mgp39 CHO Cells
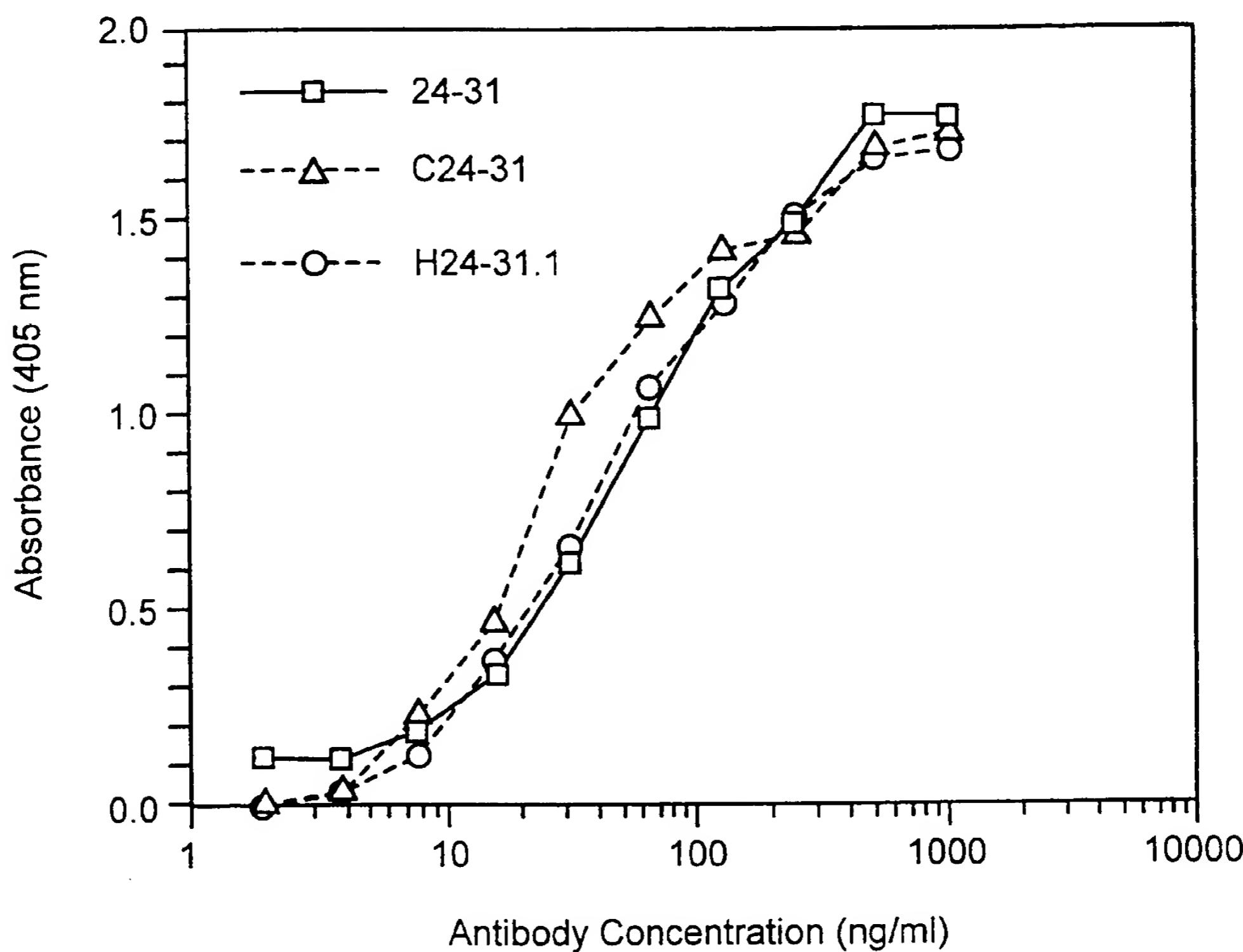
Fifty ul of 1 ug/ml solution of each anti-gp39 antibody (murine, chimeric and humanized version 1 of 24-31) was added to wells containing poly-l-lysine fixed mgp39 CHO cells. After a 2 hour incubation, the bound antibodies were detected with either goat anti-human IgG HRP or goat anti-human IgG HRP or goat anti-mouse IgG HRP. The binding capacity of each antibody was compared on a plot of absorbance vs antibody concentration.

The figure shows that the half maximal binding in ELISA is achieved at similar concentrations for all three versions, at approximately 40 ng/ml.

FIG. 10

**Competition Binding of 200 ng/ml Mouse Anti-gp39 Biotin
with Anti-gp39 Antibodies on mgp39 CHO Cells**
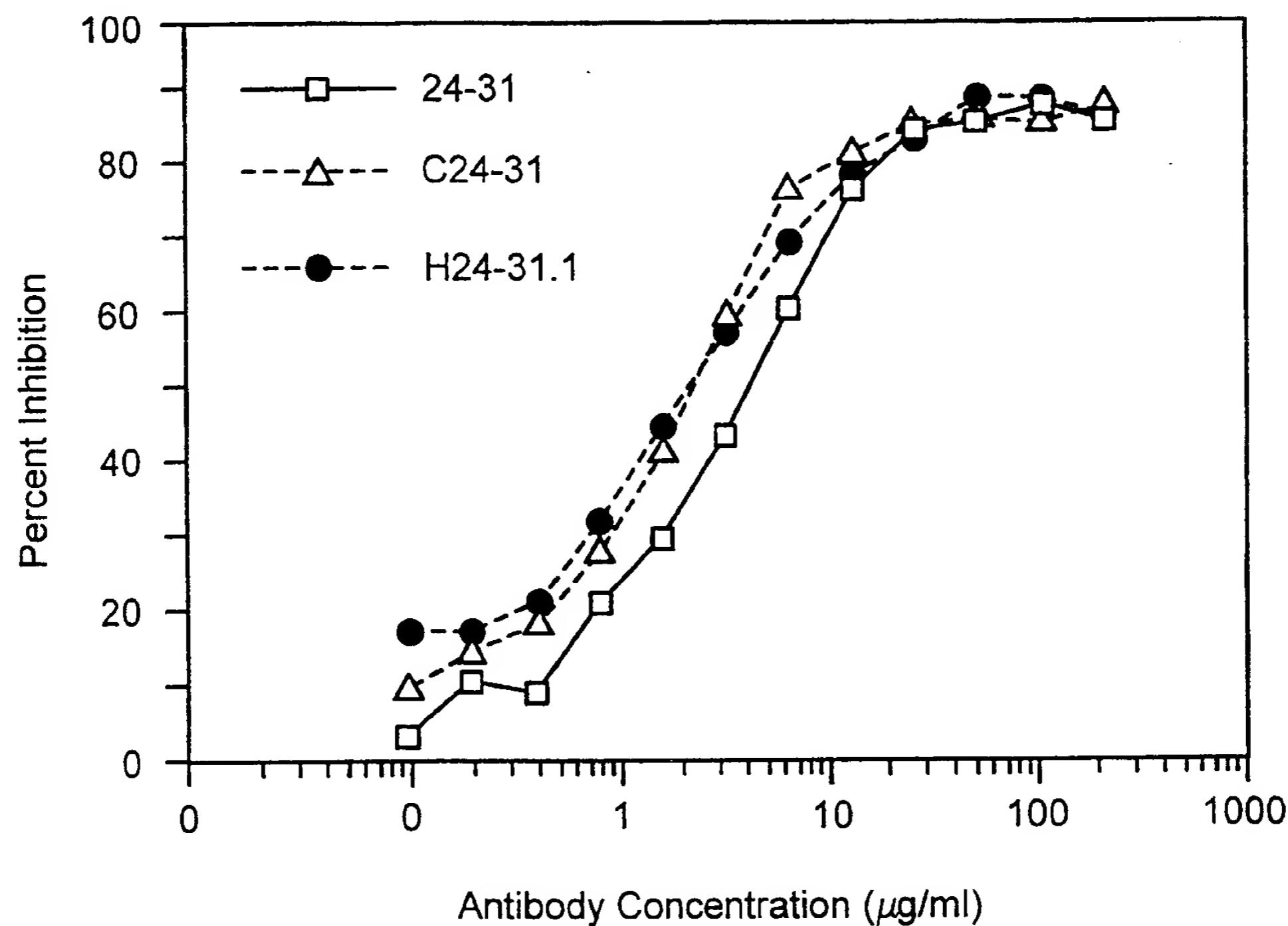
The figure shows that competition for binding to gp39 between biotin label
24-31 and the mouse, chimeric and humanized versions 1 are comparable,
possibly with the humanized version slightly better than the original antibody,
with half-maximal competition at 2 and 4 μg/ml, respectively.

FIG. 11
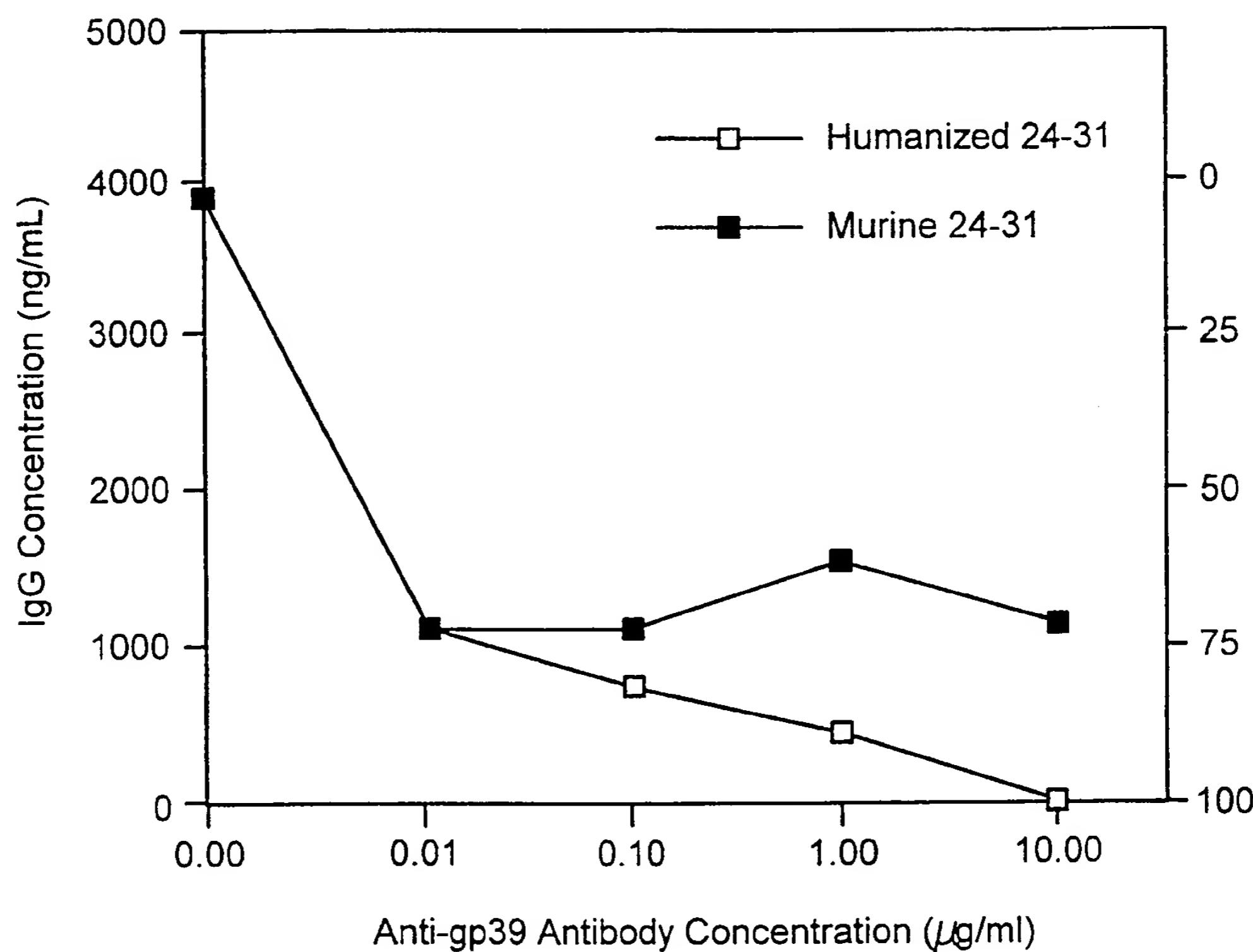
Purified, mitomycin C treated T cells were added into cell culture plates coated with anti-CD3 antibody. Autologous purified B cells were mixed with antibody at described concentrations and added to these plates in regular growth media. After 10 days the supernatant was tested for content of human IgM.

FIG. 12
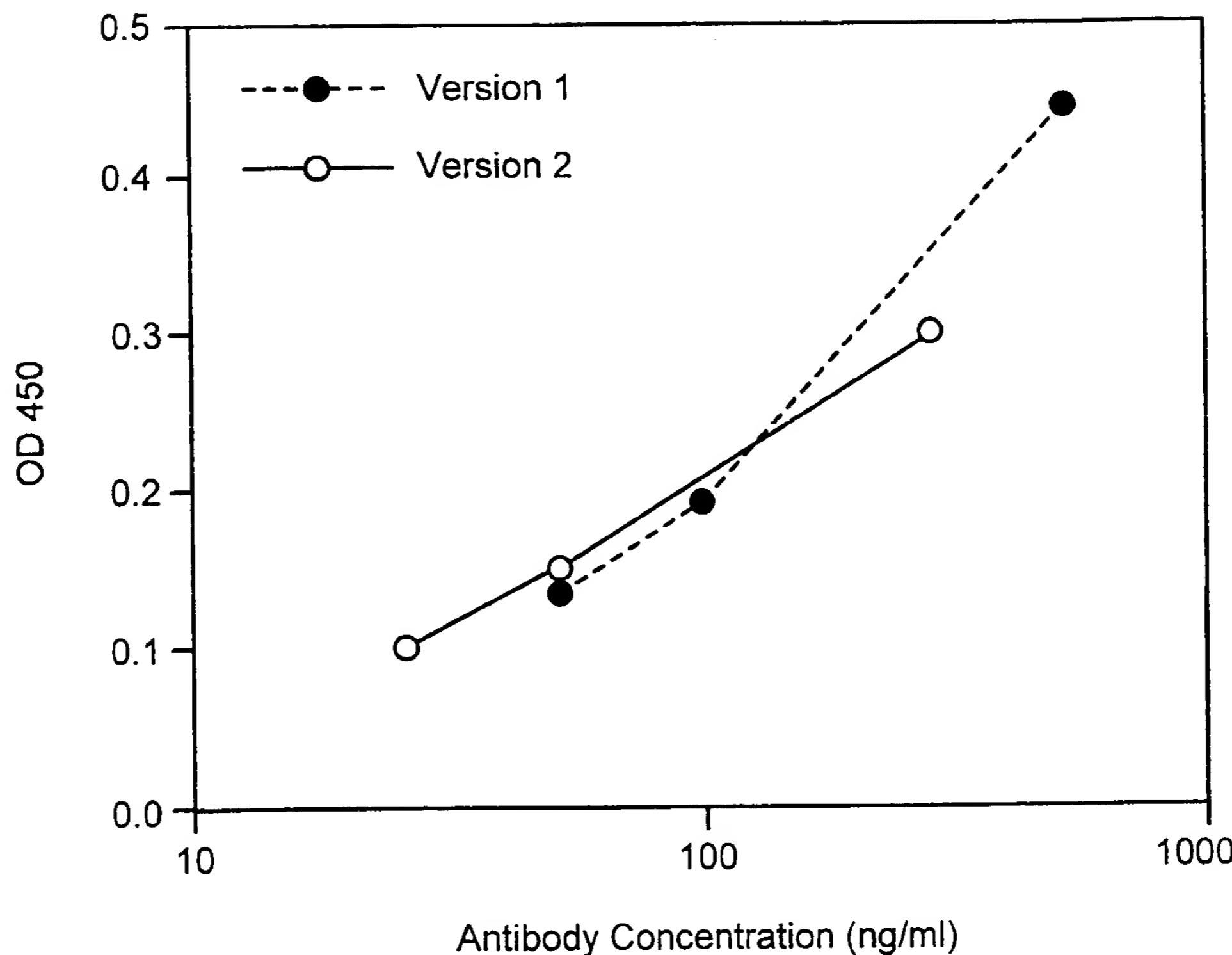
CHO cell supernatant containing humanized 24-31 version 1 and version 2 in unknown amounts, was incubated on mgp39-CHO cells for 2 hours. After a wash, the amount of bound antibody was determined. The same supernatants were tested in parallel on an ELISA plate coated with Goat α Human γ, to determine the concentration of human IgG present relative to a control of known concentration. The binding data were normalized relative to the total antibody concentration.

FIG. 13

Scatchard Analysis

y = 2.1573e-3 - 1.1750e-3x R^2 = 0.952

Kd= 8.51 nM

B/F (y-axis)

Mouse anti-gp39 (B) x10 E-11 M (x-axis)

FIG. 14

Scatchard Analysis

y = 3.5621e-3 - 1.7839e-3x R^2 = 0.943

Kd= 5.60 nM

B/F

Humanized anti-gp39 Version 1(B) x10 E-11 M

FIG. 15

Scatchard Analysis

$y = 3.0614e\text{-}4 - 7.1470e\text{-}4x \quad R^2 = 0.888$

$Kd = 14\ nM$

B/F

Humanized anti-gp39 Version 2(B) x10 E-11 M
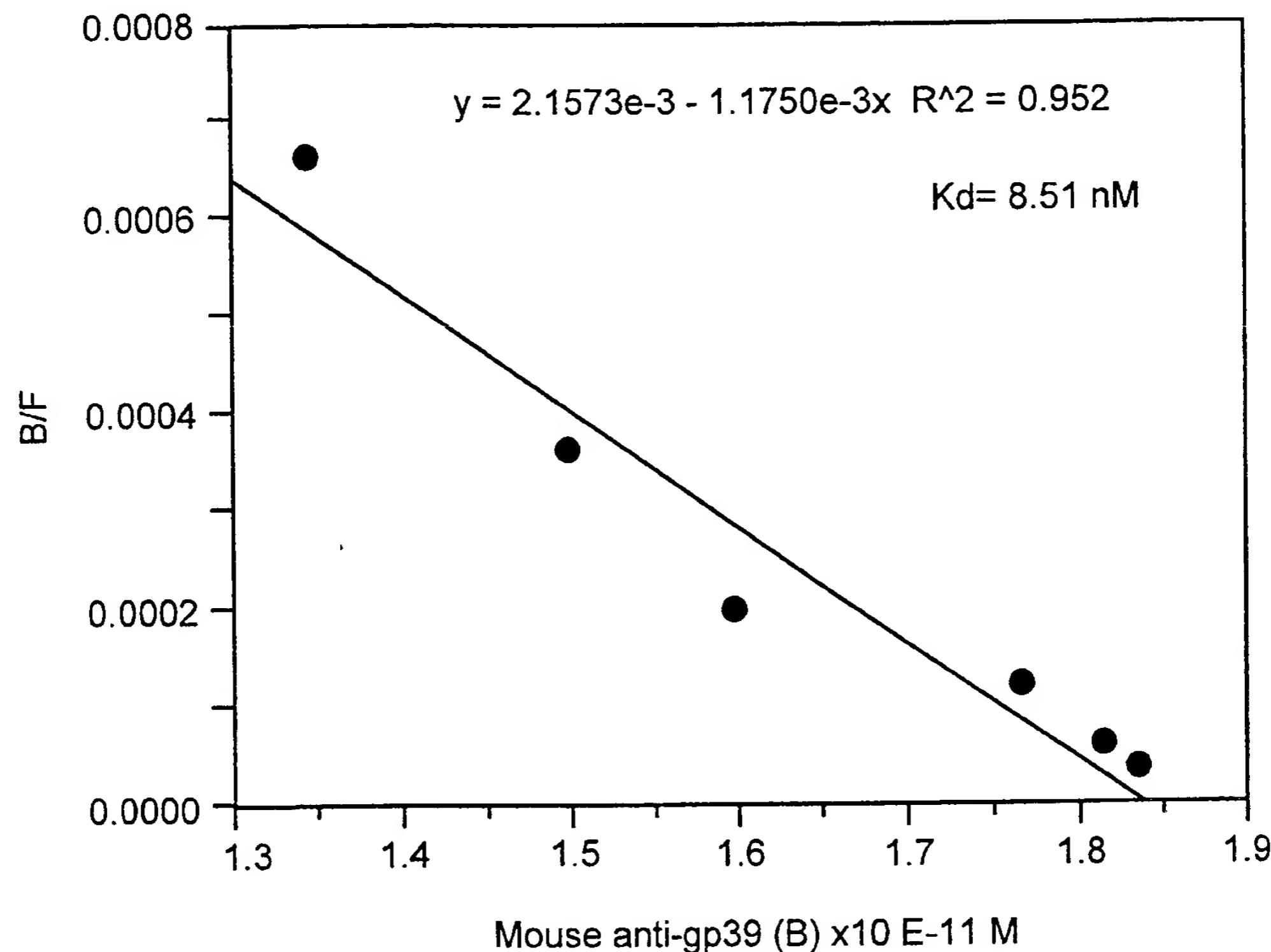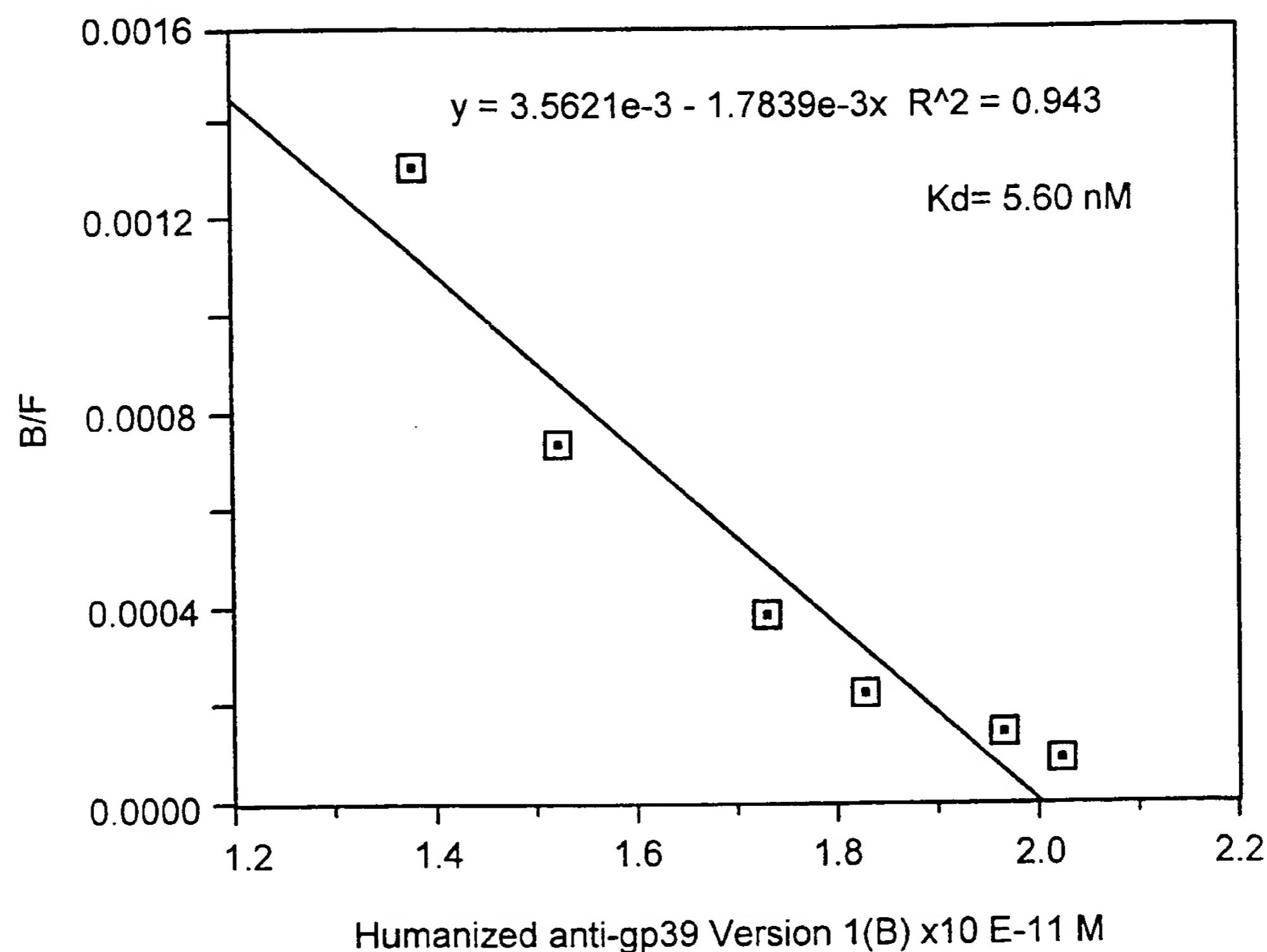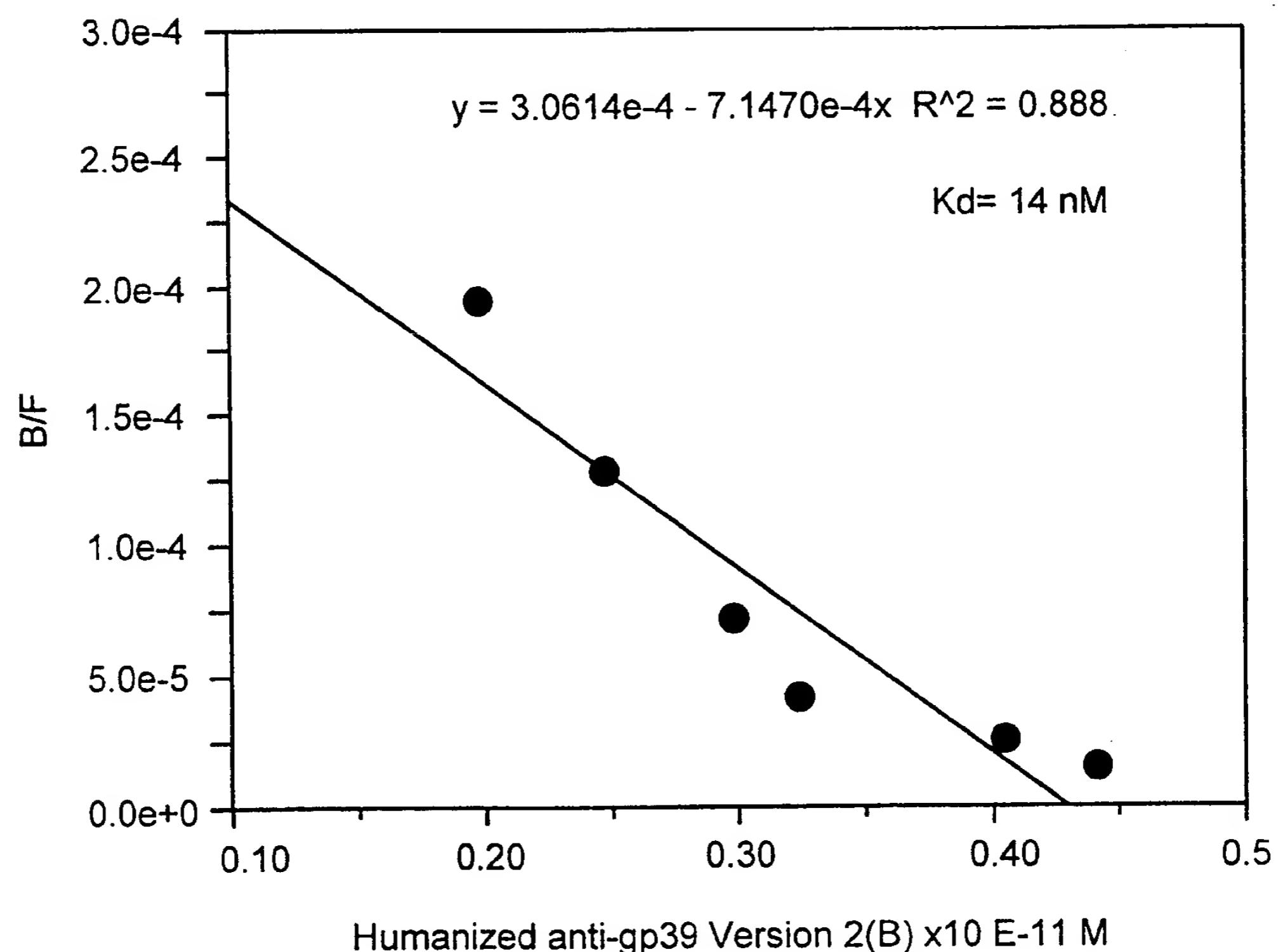
FIG. 16

FcRI Binding of H24-31.1 in the Presence of gp39-CD8 Fusion Protein
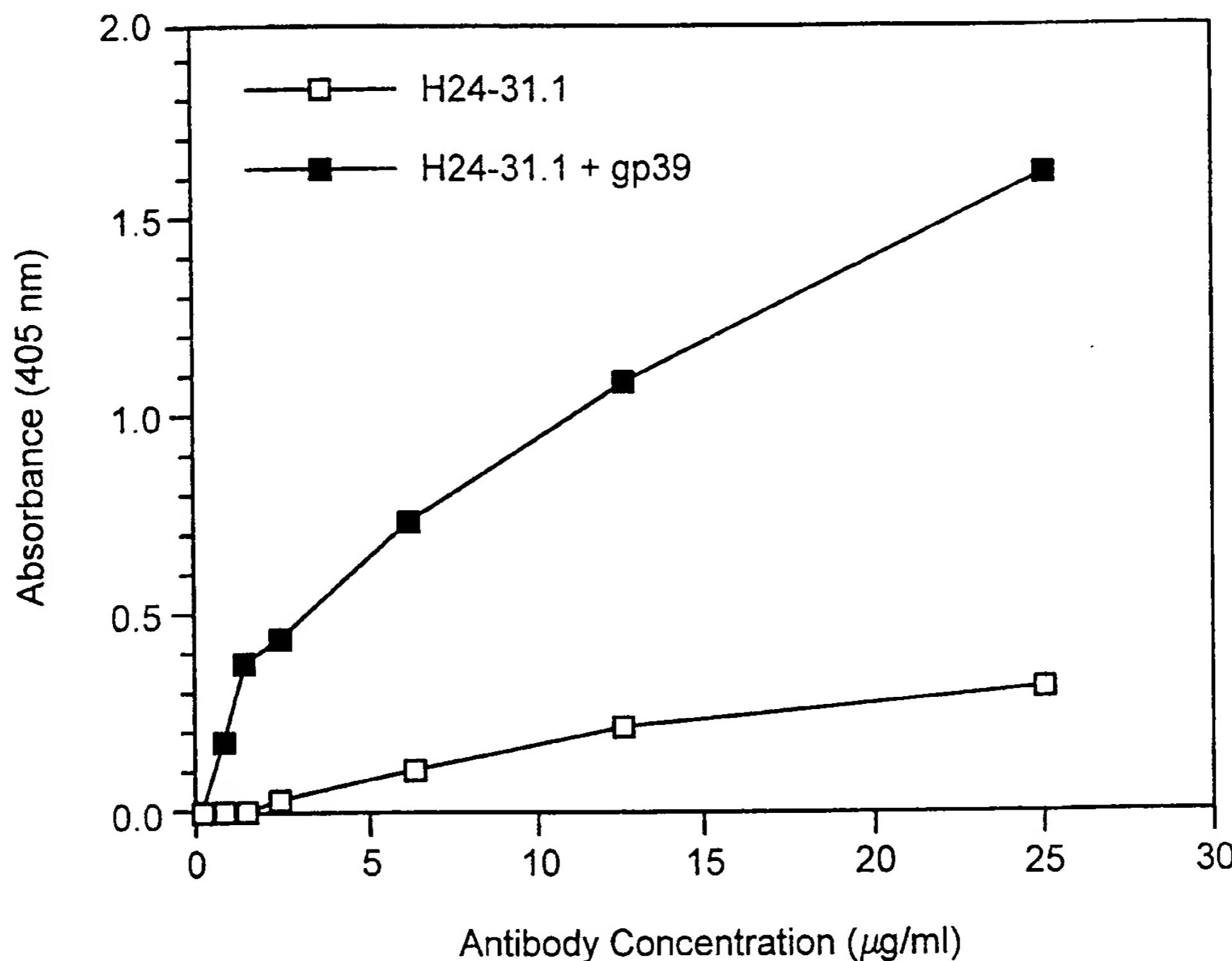
The figure shows that H24-31.1 only binds Fc receptor I when complexed to antigen.

FIG. 17

FcRII Binding of H24-31.1 in the Presence of gp39-CD8 Fusion Protein
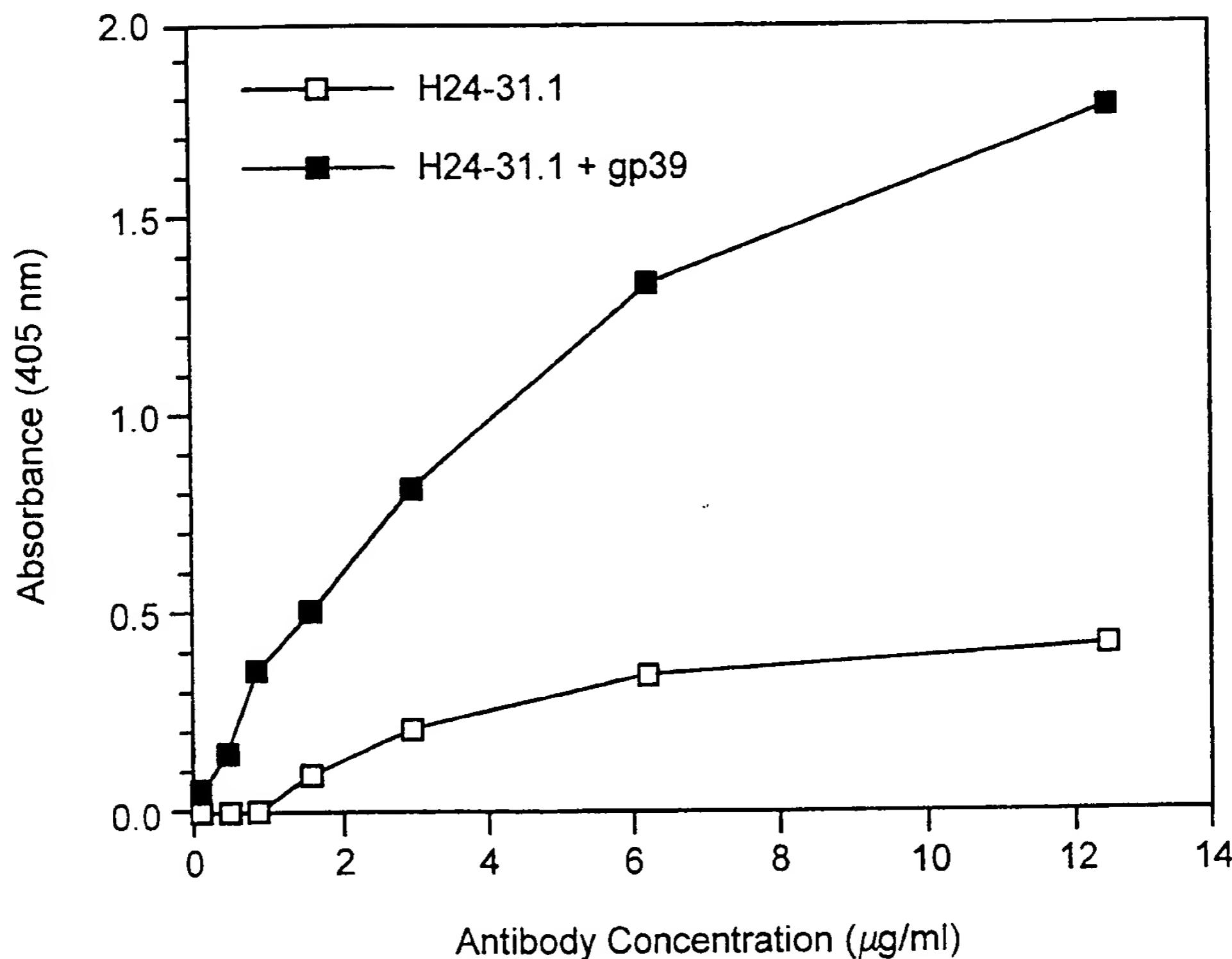
The figure shows that H24-31.1 only binds Fc receptor II when complexed to antigen.

FIG. 18

Complement Dependent Cellular Cytotoxicity using Alamar Blue
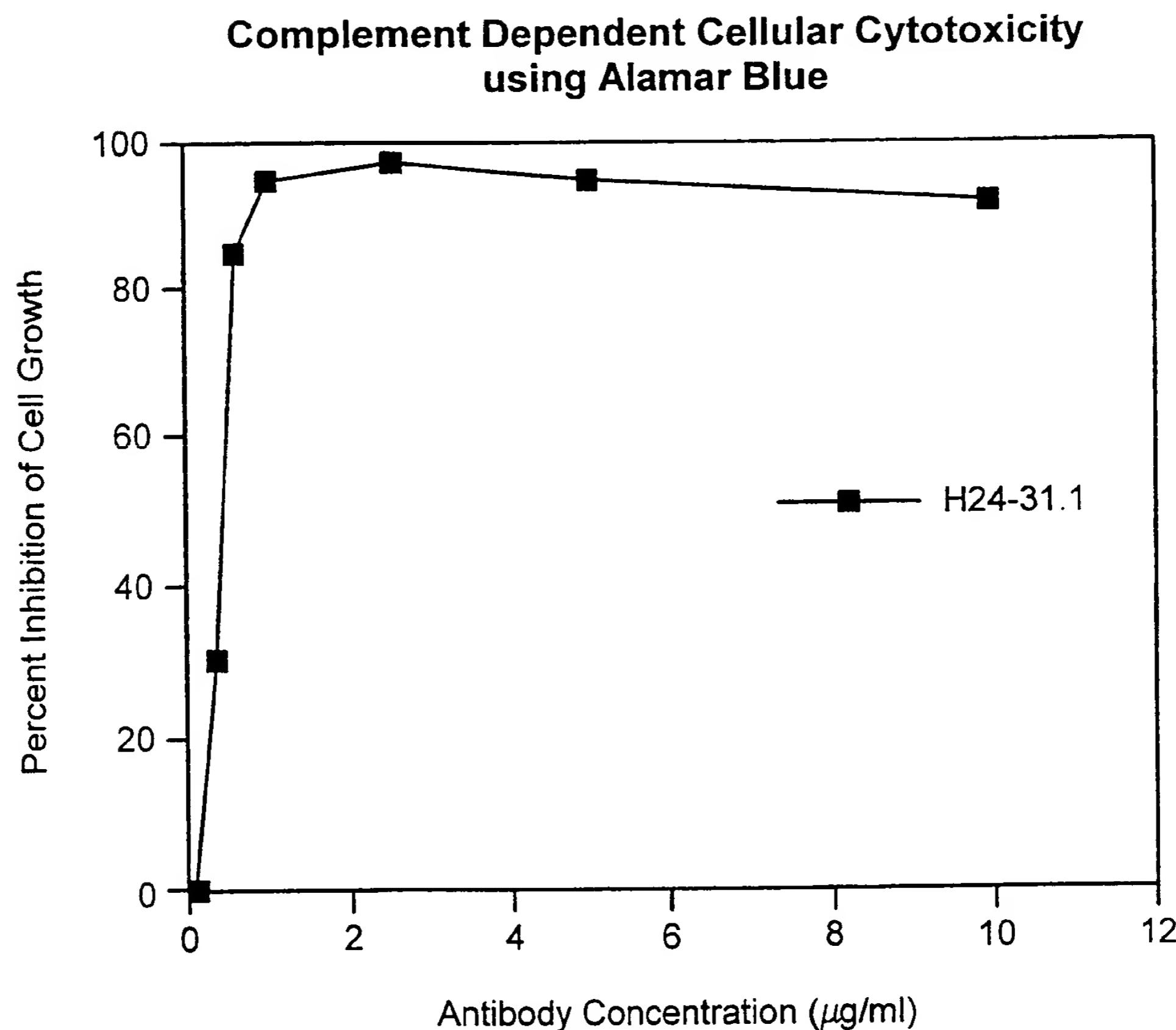
The figure shows that H24-31.1 inhibits 50% of cell growth at approximately 0.5 μg/ml.

FIG. 19

Determination of C1q binding of gp39+CHO bound H24-31.1 (Humanized 24-31 version 1)

gp39+CHO cells labeled with various concentrations of H24-31.1 were incubated in an excess amount of human C1q (complement factor 1) and subsequently incubated with FITC labaled goat anti-human C1q. The samples were analyzed by flow cytometry to determine the relative labeling of the cells with C1q.

C1q Binding of H24-31.1
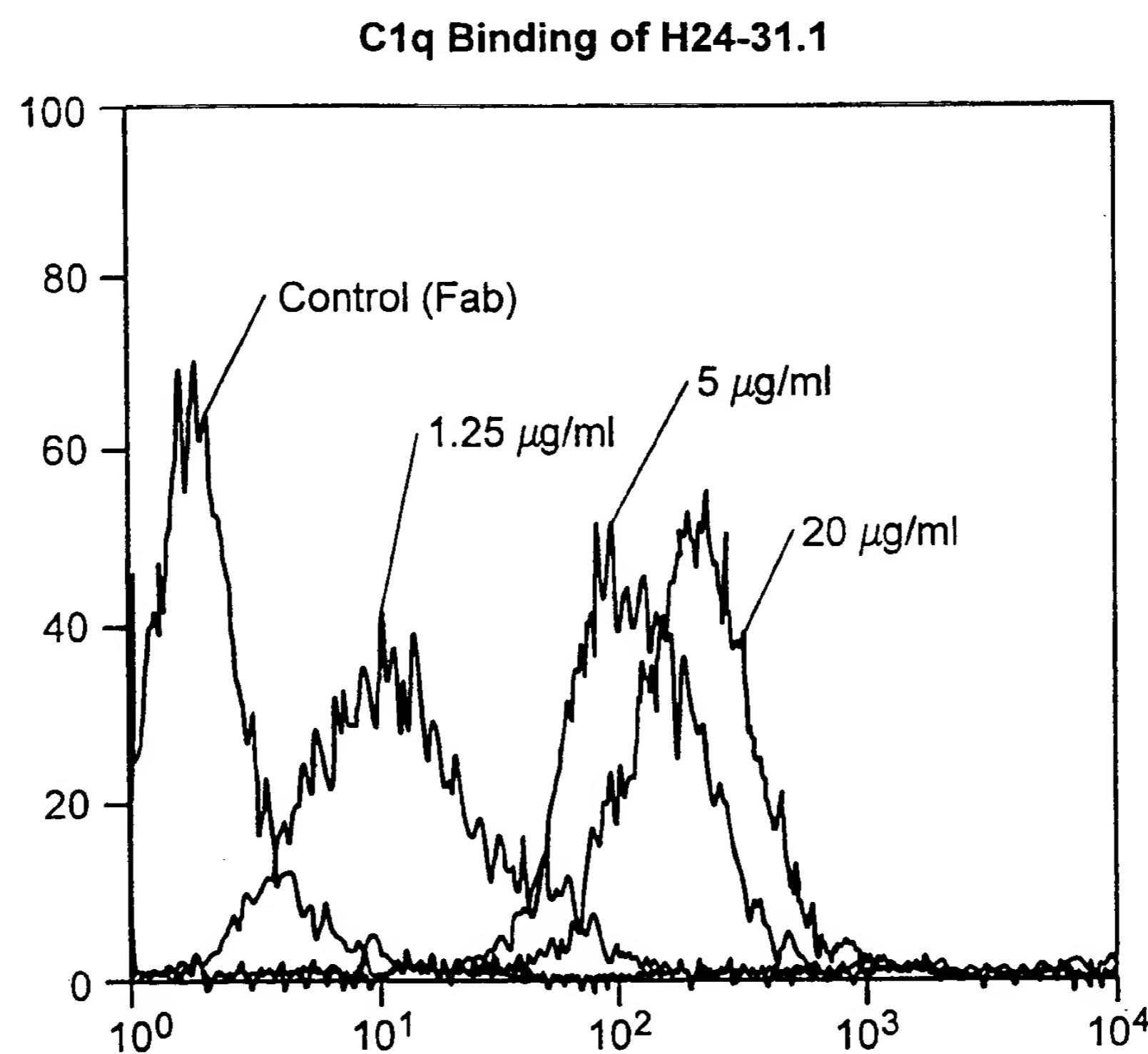
The figure shows that labeling of the cells with C1q was dependent on the antibody concentration, and that an H24-31.1 Fab fragment (no C1q binding) did not bind C1q.

FIG. 20

Effect of H24-31.1 on T cell responses to a T cell dependent recall antigen.

Growth
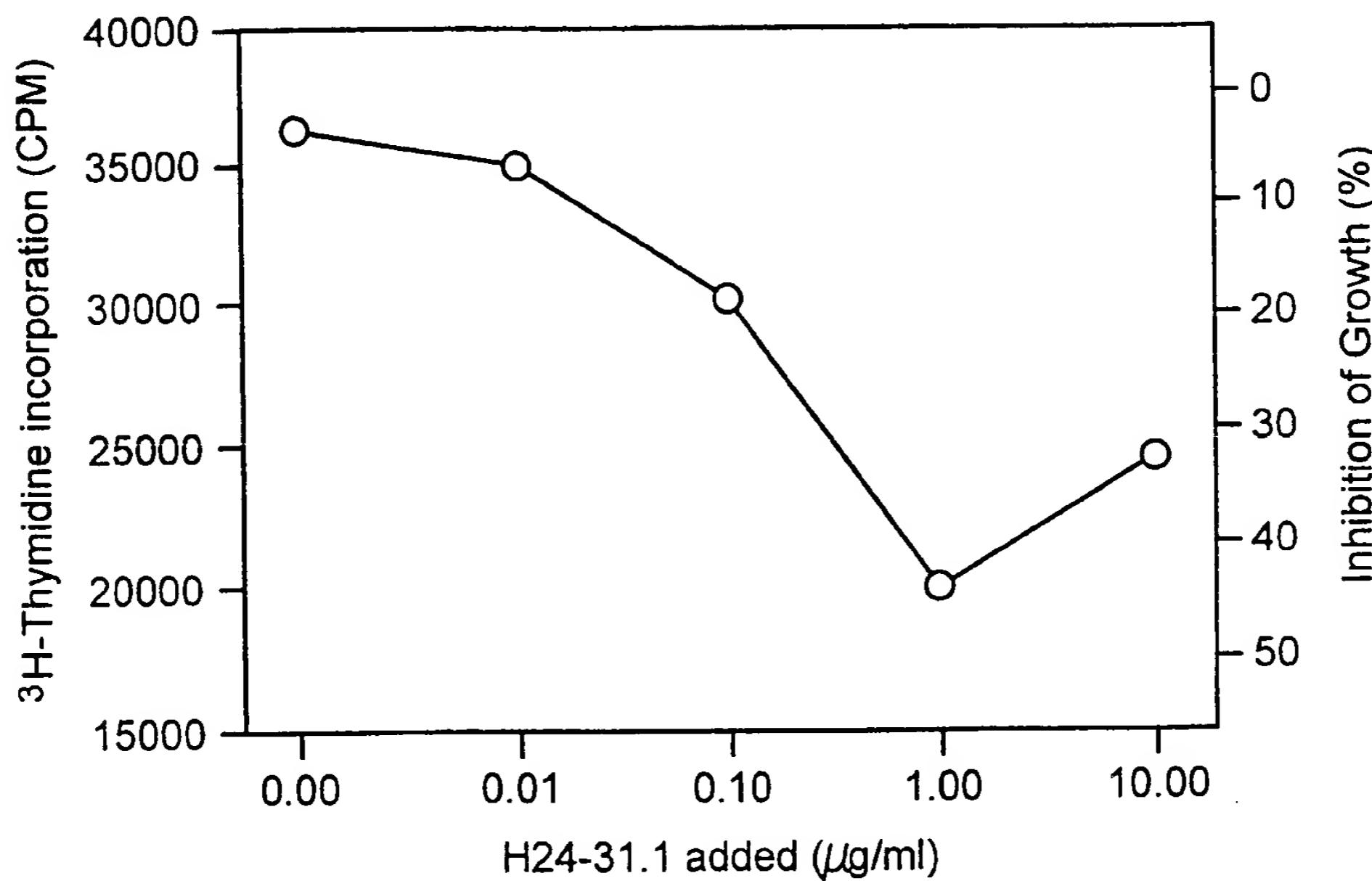
IL-2 Production
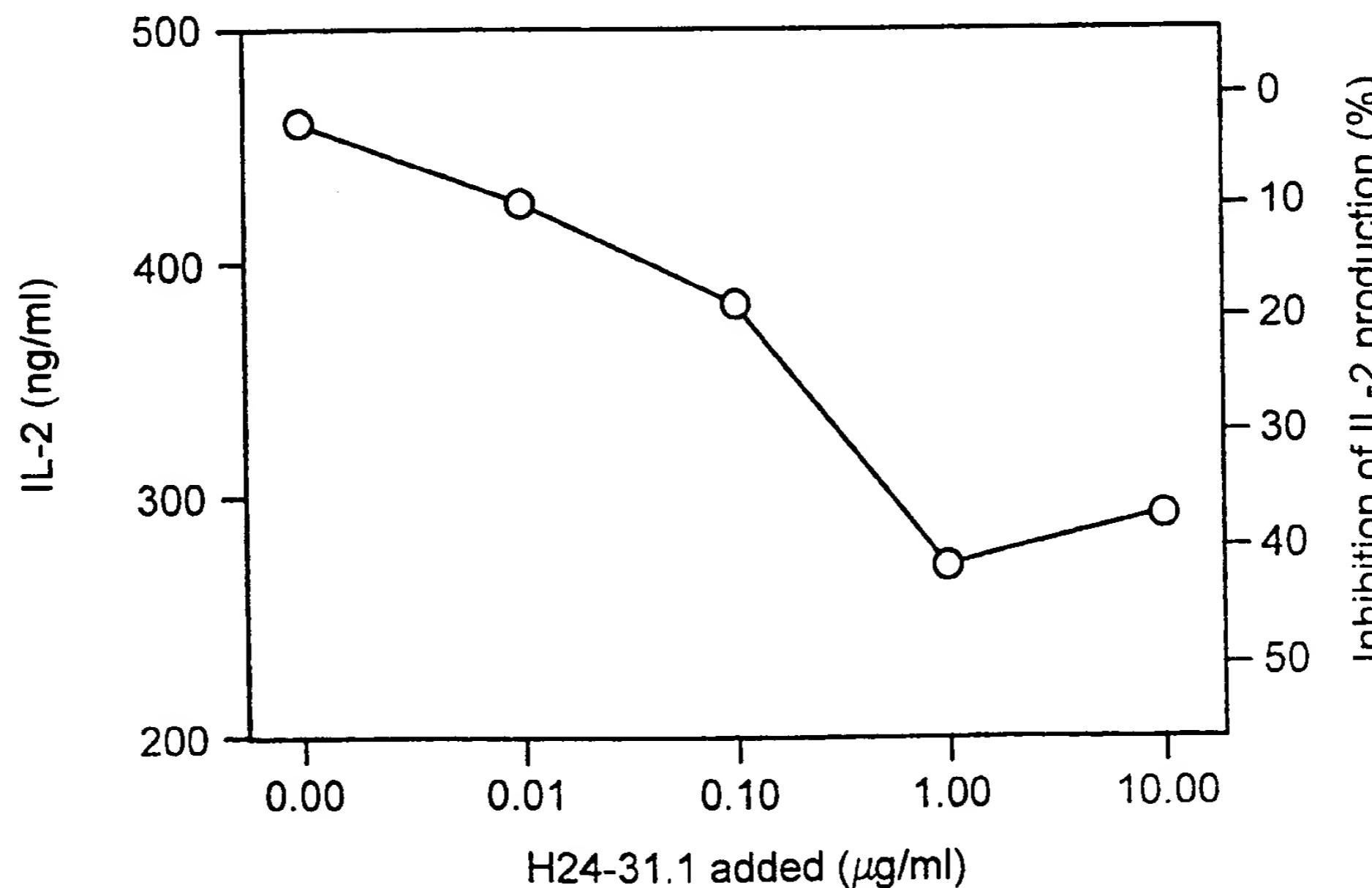
The figures show that TT induced growth and IL-2 production was inhibited maximally, around 40%, by ≥ 1µg/ml H24-31.1.

FIG. 21

Effect of H24-31.1 on T cell dependent B cell differentiation.

**Inhibition of T Cell Dependent IgG Production by
Anti-gp39 Antibodies**
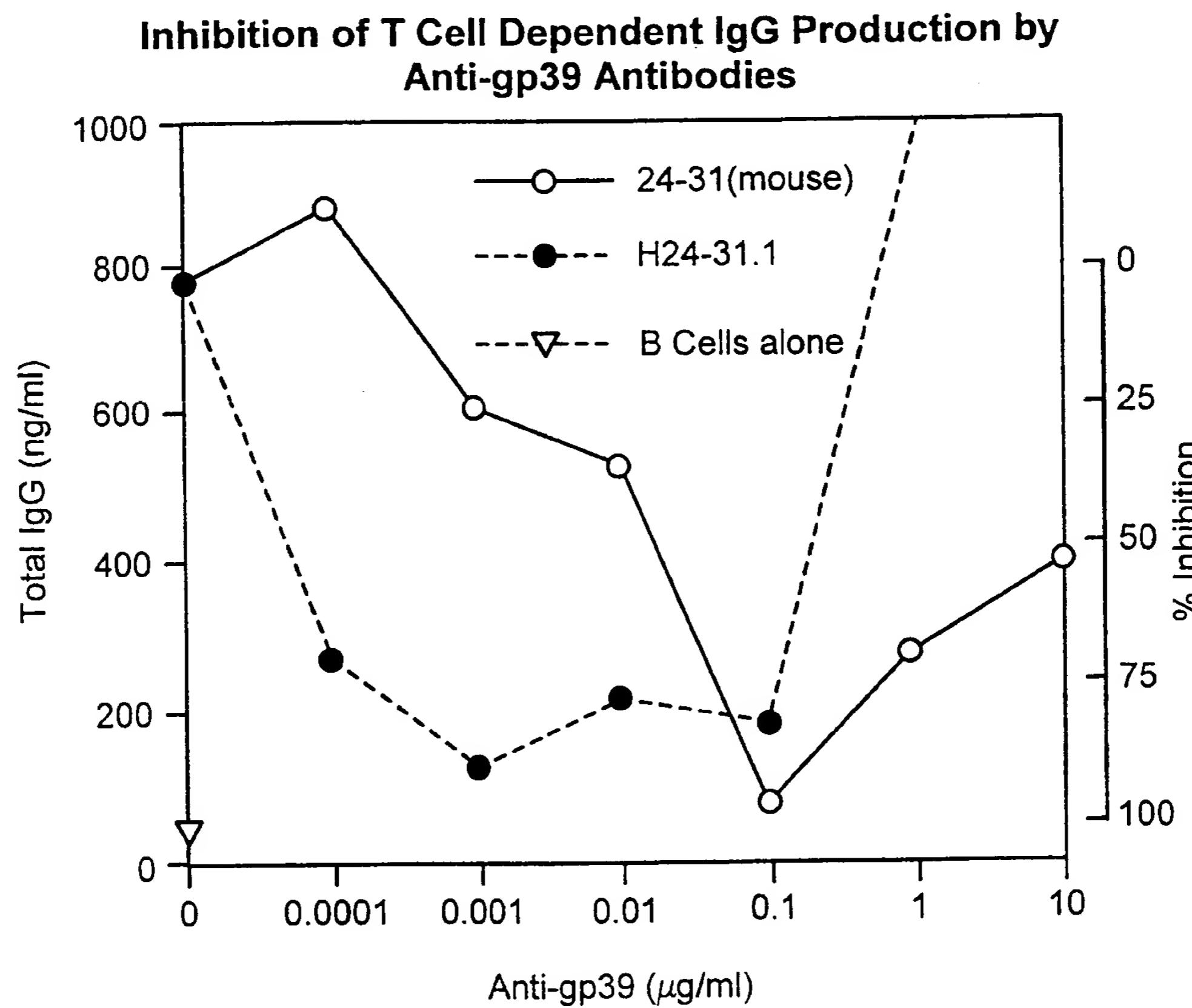
The figure shows that H24-31.1 inhibits T cell dependent IgG production approximately 85% at 1 ng/ml. The apparent increase in IgG production seen at higher concentrations is due to the inability of this test to distinguish H24-31.1 from antibody produced by the B cells.

FIG. 22

Effect of H24-31.1 Fab on T cell dependent B cell differentiation.

**Anti-gp39 Antibody Inhibition of T Cell Dependent
B Cell IgG Production**
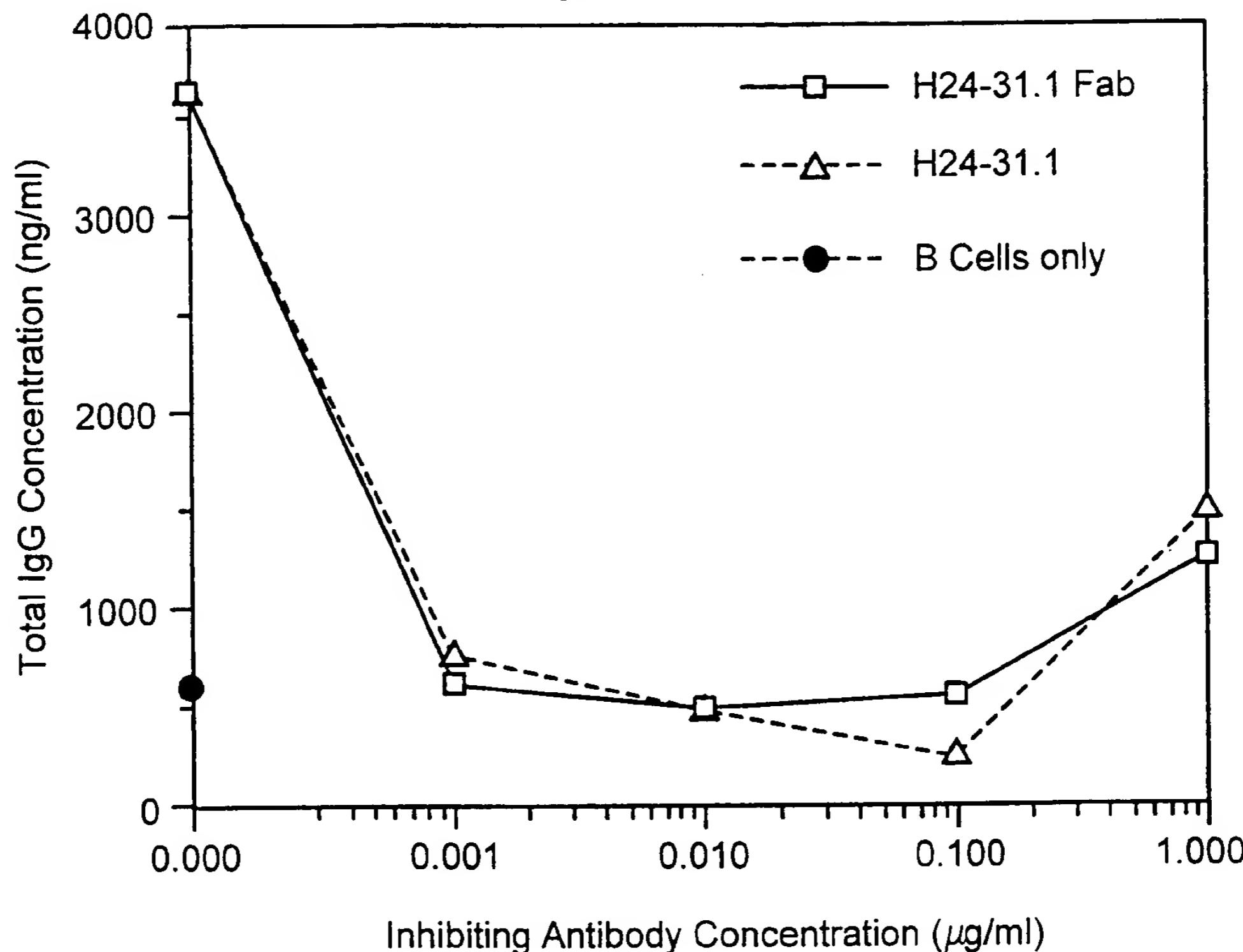
The figure shows there was no difference in the inhibition of IgG
production between the whole H24-31.1 antibody and the Fab fragment.

FIG. 23

Effect of H24-31.1 on generation of antigen specific B cells responses
to a T cell dependent recall antigen.

Human spleen cells primed with tetanus toxoid for 3 days in vitro,
were transferred to SCID mice at a concentration of approximately 1×10^7
cells/SCID. After 6 six days the resulting hu-SPL-SCID mice were injected
with PBS (group 1) or with 300 μg H24-31.1 (groups 1 and 2). The following
day the Hu-SPL-SCIDs were all boosted with tetanus toxoid. The mice in
group 3 received 2 further injections of 300 μg H24-31.1 each with 3 days
interval. The mice were bleed at various timepoints and the levels of human
IgG anti-tetanus toxoid titers determined by ELISA.

Anti-TT titer in 24-31-Hu-SPL-SCID
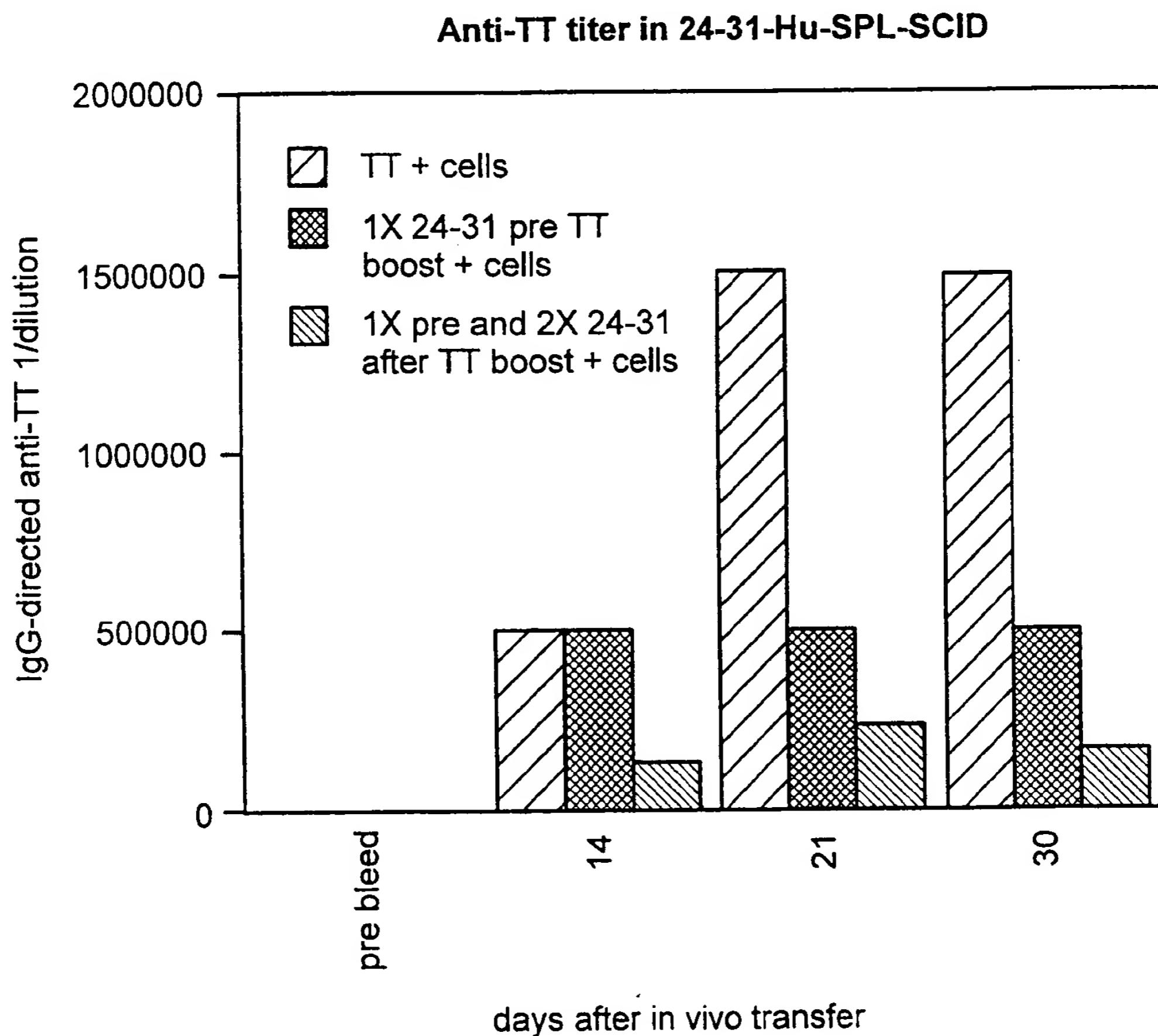
The figure shows that injection of H24-31.1 inhibited generation of tetanus
toxoid specific responses by approximately 90% (group 3). Levels of total
human IgG was comparable between the three groups.

FIG. 24

## Proliferation of Human B Cells by Soluble gp39-CD8
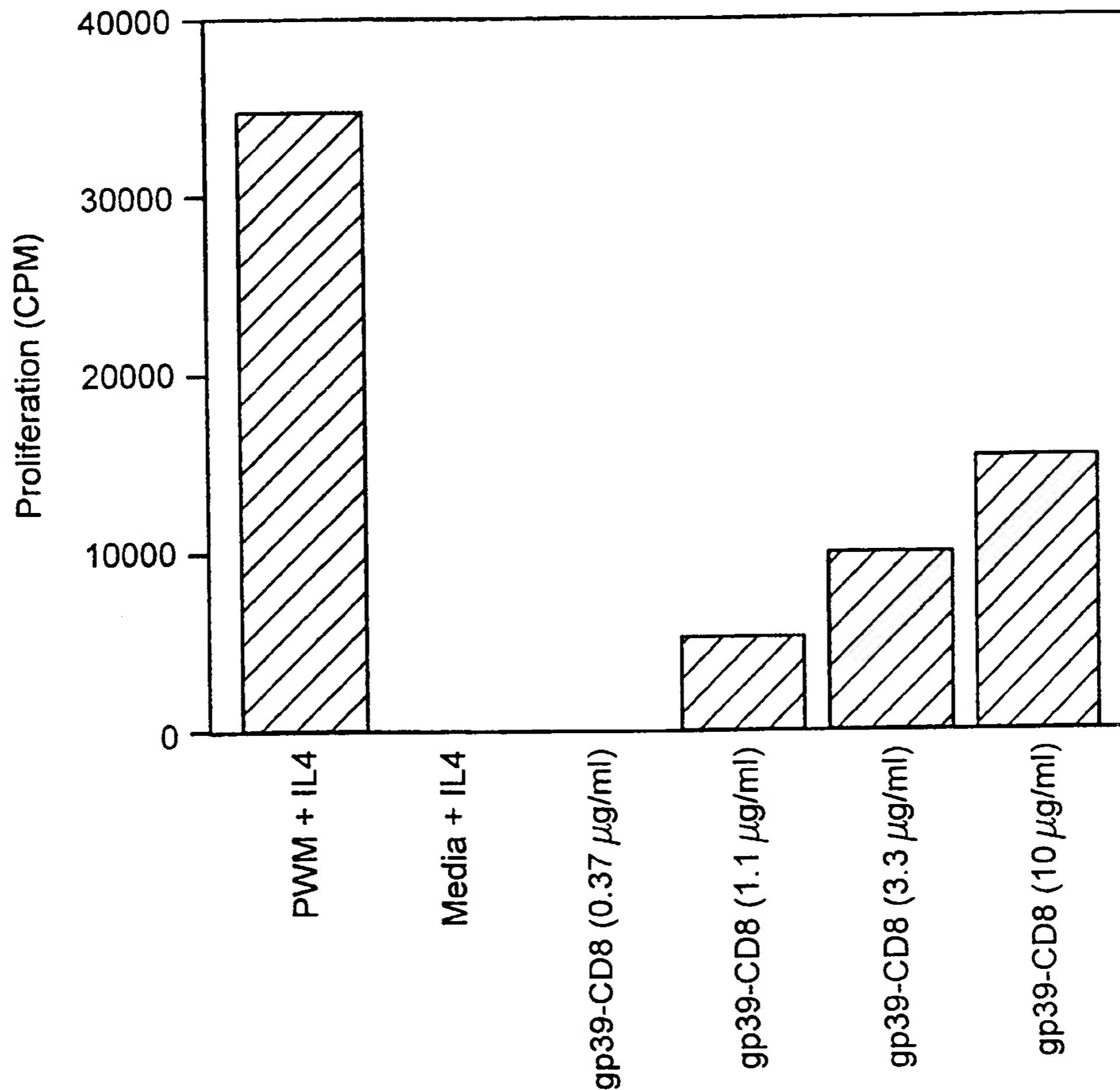
Different concentrations of CD8-gp39 were incubated with enriched human B cell plus IL-4, in a 4 day proliferation assay. The CPM obtained in B cells plus IL-4 cultures without gp39-CD8 was used as the background and subtracted from the total CPM of test culture stimulated with gp39-CD8. Cells cultured with PWM plus IL-4 served as positive control for B cell proliferation. CPM represents the mean CPM of samples tested in triplicates.

FIG. 25

Inhibition of B-Cell Proliferation by H24-31
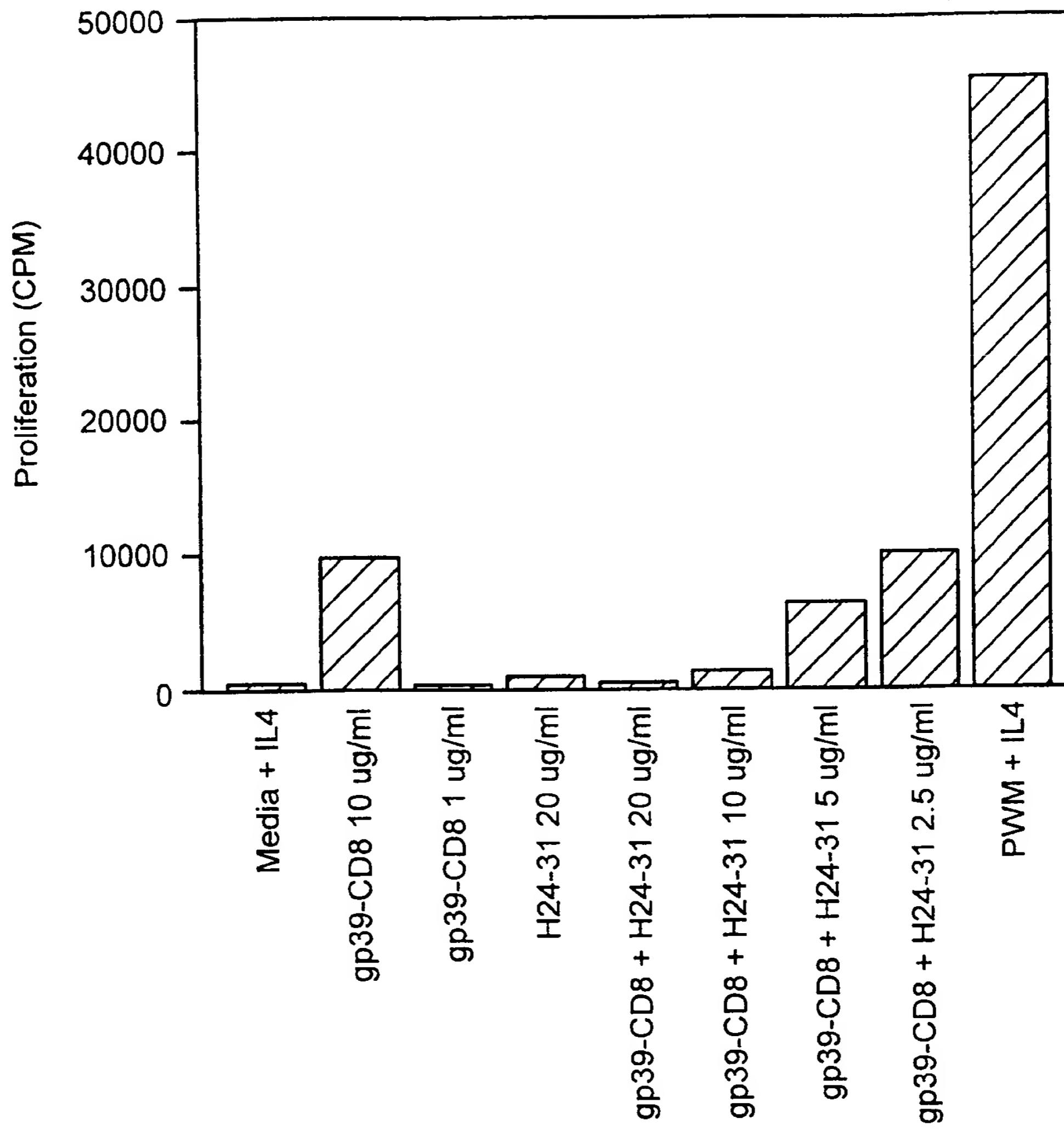
Different concentrations of H24-31.1 antibody were incubated with B cells (1x10^5) cultured with IL-4 (1000 U/ml) and 10 μg/ml of gp39-CD8 in a 4 day proliferation assay. Cells cultured with PWM plus IL-4 served as positive control for B cell proliferation. CPM represents the mean CPM of samples tested in triplicates.

FIG. 26

Blocking of CD40-Ig binding to gp39 by humanized versions of 24-31

—□— Control

---○--- Version #2
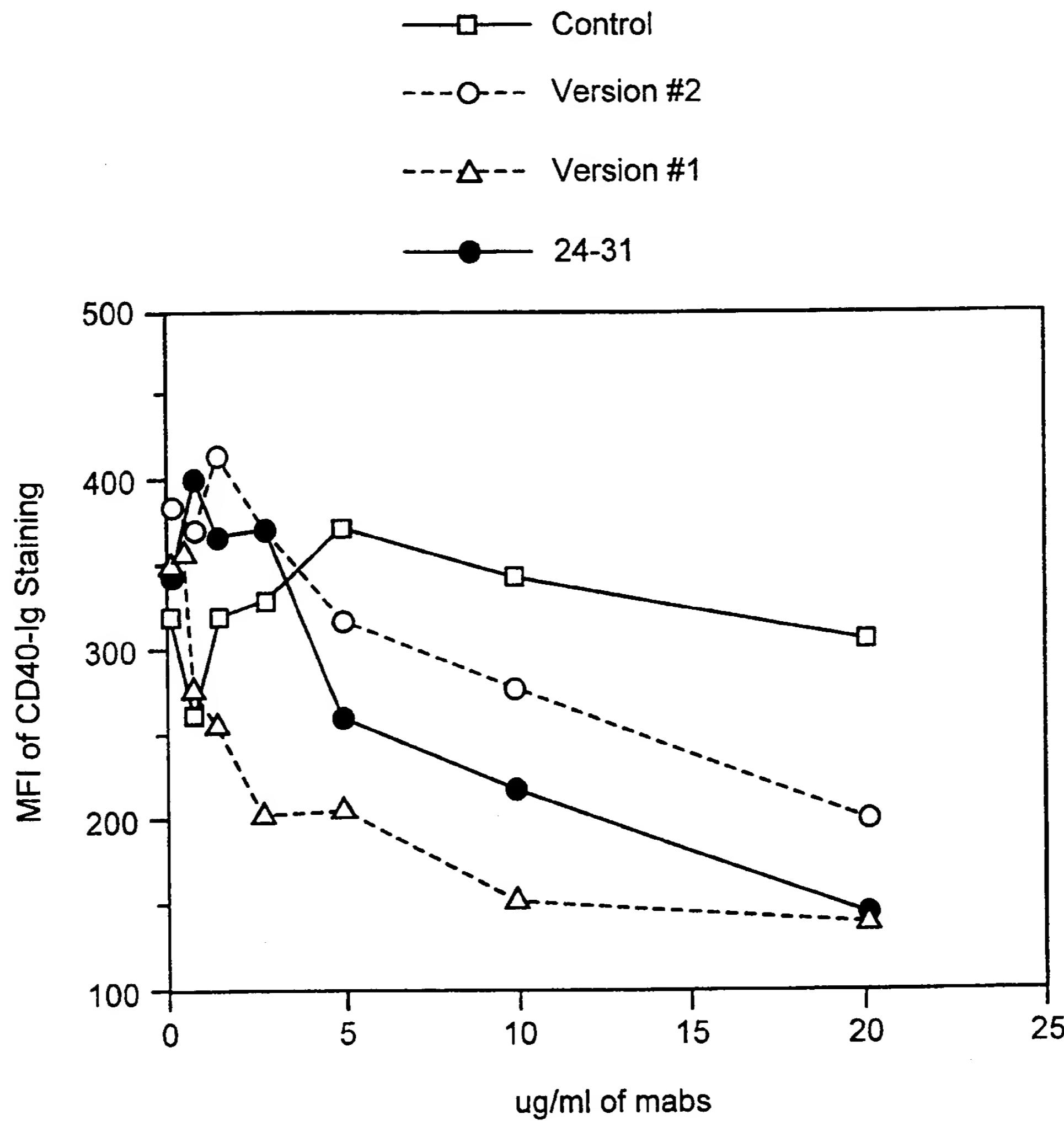
---△--- Version #1

—●— 24-31

This figure illustrates that the murine and humanized versions of 24-31 block binding of CD40-Ig.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/18163

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :Please See Extra Sheet.

US CL   :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, CA, EMBASE, MEDLINE, WPI

search terms: gp39, CD40L, cd40 ligand, 24(w)31, autoimmun?, transplant?, graft?

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,474,771 A (LEDERMAN ET AL.) 12 December 1995, see entire document.	1-6, 10-16, 25-29
X	WO 94/04570 A1 (SCHERING CORPORATION) 03 March 1994, see entire document.	1-6, 10-16, 25-29
X	WO 95/06666 A1 (TRUSTEES OF DARMOUTH COLLEGE) 09 March 1995, see entire document.	1-34

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 NOVEMBER 1998	22 DEC 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PHILLIP GAMBEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39.395; CO7H 21/04; CO7K 16/00, 16/18, 16/28; C12N 5/10, 15/13; C12P 21/08

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/1301., 133.1, 141.1, 143.1, 144.1, 152.1, 154.1, 173.,1; 435/451. 452, 326, 328, 332, 334, 343, 343 .1, 343.2; 530/387.1, 387.3, 388.1, 388.2, 388.33, 388.7, 388.75; 536/23.1, 23.4, 23.5, 23.53

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

424/1301., 133.1, 141.1, 143.1, 144.1, 152.1, 154.1, 173.,1; 435/451. 452, 326, 328, 332, 334, 343, 343 .1, 343.2; 530/387.1, 387.3, 388.1, 388.2, 388.33, 388.7, 388.75; 536/23.1, 23.4, 23.5, 23.53